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Volume XVI JULY, 1955 No. 60

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VOL. XVI JULY, 1955 No. 60

Studies on Arthritis in Swine
I. Experimental Erysipelas and Chronic Arthritis in Swine

DENNIS SIKES, D.V.M., Ph.D.; GEORGE M. NEHER, Ph.D.; L. P. DOYLE, D.V.M., Ph.D.

Lafayette, Indiana

SWINE erysipelas is a disease in which great variations occur. The morbidity and the mortality, as well as the clinical and pathological manifestations, are extremely variable. The number of animals that recover spontaneously varies a great deal in different herds. Moreover, it is often futile to attempt to predict whether or not the disease will recur on any farm.

The causative organism is assigned to the family Corynebacteriaceae and genus Erysipelothrix according to Bergey.[3] Pasteur and Dumas[29] described the organism, and Loeffler[26] is credited with the first observation of the organism in swine. He observed the organism in the blood vessels of a pig which apparently had died from swine erysipelas and thought it was similar to Koch's *Erysipelothrix muriseptica*. Later, Rosenbach[28] suggested the names *Ery. muriseptica*, *Erysipelothrix porci*, and *Erysipelothrix erysipeloides* for the mouse, pig, and human organisms, respectively. Konst[15] has suggested the possibility of two variants of *Erysipelothrix rhusiopathiae*, the highly virulent one prevalent in Europe, and a less virulent one in North America. The observations of Breed[4] do not support this concept, in that the disease is spreading in North America and is of great economic importance. Perhaps the more virulent strains have been imported in swine originating in Europe. Konst[15] further suggests that Koch's *Ery. muriseptica* may be considered as an attenuated strain of *Ery. rhusiopathiae* because of similar morphological, serological, and immunological characteristics, and both are equally pathogenic for small animals. Prettner,[25] and Rickman[27] were unable to differentiate between the three species described by Rosenbach.[28] The relationships of the microorganisms isolated from man, sheep, chickens, turkeys, and swine have been studied by Beaudette and Hudson,[2] Klauder and Harkins,[13] and Van Roekel *et al.*[37] who were not able to show any significant cultural differences between strains.

The characteristics of *Ery. rhusiopathiae*, according to several investigators,[1, 7, 11, 20, 22, 29, 31, 34, 35, 39] are varied. Karlson and Merchant[11] used 60 different strains of *Ery. rhusiopathiae* and concluded acid was produced readily from dextrose, lactose, galactose, and levulose. A delayed reaction occurred on mannose and cellobiose. No acid formation was observed on arabinose, xylose, rhamnose, maltose, melibiose, sucrose, trehalose, raffinose, melizitose, dextrin, starch, inulin, amygdalin, salicin, glycerol, erythritol, adonitol, mannitol, sorbitol, dulcitol, or inositol.

Natural outbreaks of *Ery. rhusiopathiae* infections have been recorded in sheep, cattle, horses, turkeys, ducks, peacocks, pheasants, and farm-raised mink.[5, 9, 17, 24] Also, infections in man have occurred from handling fish, from infected meat in slaughterhouses, as well as in workers in bone button factories and in veterinary students.[13, 19, 21]

The economic importance of swine erysipelas and chronic arthritis in swine has been emphasized for more than a half century in Europe. It has been of great concern in the United States during the last two decades only. Arthritis has rated a second or third as the cause of condemnation of swine carcasses at federally inspected meat packing plants for the last seven years. According to the annual report of the Bureau of Animal Industry, U. S. Department of Agriculture (B.A.I., U.S.D.A.),[38] the number of carcasses condemned for arthritis in 1952 was 22,196. This represents only a portion of the economic loss due to the disease. Arthritis often has a marked stunting effect on growing pigs.

This investigation was supported in part by a research grant A–45(C2) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

Taken from thesis, "The Pathological Effects of the Bacterium *Erysipelothrix rhusiopathiae* on Swine," submitted to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August, 1954. Present address (Sikes): Department of Pathology and Parasitology, School of Veterinary Medicine, University of Georgia, Athens.

Submitted as journal paper No. 837, Agricultural Experiment Station, Purdue University, Lafayette, Ind.

Attempts to reproduce swine erysipelas in swine have met with inconsistent and limited success. Creech [6] exposed 22 pigs which were negative to the agglutination test for *Ery. rhusiopathiae*. The methods of exposure were by feeding and by intravenous, subcutaneous, and intraperitoneal inoculation. Five pigs died and the organism was isolated from the spleen, liver, kidneys, and blood. Six pigs survived after showing symptoms of the disease while 11 pigs showed no evidences of the disease. Schoening *et al.*[23] inoculated 10 hogs with splenic emulsions and cultures taken from swine which died of erysipelas. Sixty per cent developed acute swine erysipelas while 40 per cent became chronically affected.

Ward [28] isolated *Ery. rhusiopathiae* from 16 of 22 sets of legs of arthritic pigs. He inoculated 1 pig intravenously with a suspension of the joint exudate and later gave two additional injections of the organisms. Later, the pig developed arthritis with extensive bone and connective tissue proliferation similar to the natural disease. The lesions were negative when examined bacteriologically. Intravenous inoculation of adult hogs yielded negative results.

Collins and Goldie [5] produced advanced chronic polyarthritis in 4 shoats which received repeated intravenous injections and in 4 shoats which received repeated subcutaneous injections but not in shoats receiving only one or two intravenous exposures of 2 cc. of a 24-hour broth culture of the organism.

Usdin *et al.*[26] reported the use of repeated injections of *Ery. rhusiopathiae* and a filtrate from the organisms grown in infusion broth for forty-eight hours. No arthritis developed in the group receiving the filtrate. Of 8 shoats receiving repeated injections of culture, only 1 developed severe polyarthritis which persisted for seven months. A less severe form of arthritis developed in the remaining 7 animals. The symptoms were maximum at four to five months, and recovery occurred by nine months.

Kobe,[14] in reviewing Loeffler's original work on swine erysipelas, reported that Loeffler injected 13 hogs with tissues from mice which had been infected with swine erysipelas and with cultures of *Ery. rhusiopathiae*. Only 1 of 13 pigs showed any significant symptoms, and the necropsy findings suggested it died of some cause other than from infection resulting from the injection of culture.

Numerous antigens have been prepared for the serological diagnosis of *Ery. rhusiopathiae* infections. The tube agglutination test developed by Schoening *et al.*,[20] when properly conducted and interpreted, shows some promise. Karlson and McNutt [10] have reported on a microscopic agglutination test which also shows promise. In both of these serological tests, the choice of the strain used and the manner of preparation of the antigen seem to be the important factors.

The object of this series of experiments was to study the pathological changes caused by *Ery. rhusiopathiae* in swine and their relationship to arthritis. Observations by Doyle [8] have indicated that naturally occurring arthritis in swine has some pathological features similar to those observed in the human being. It may be that findings in arthritis in swine will be of value in research on human arthritis.

Materials and Methods

A total of 248 swine was used in these experiments; 8 had naturally occurring field cases of swine erysipelas, and 240 were experimental animals. The experimental swine came from four sources. All were vaccinated against hog cholera by using hog cholera antiserum and virus at weaning time, except 14 shoats which were used in an experiment to compare the effects of *Ery. rhusiopathiae* on vaccinated and unvaccinated shoats. All animals were between 3½ and 5 months of age, weighed between 75 and 175 lb. and were in excellent health. The first group of 21 hogs were Duroc and Poland China grades, weighing between 150 and 175 lb. each. A complete history of these 21 hogs was not available. The second group of 40 pigs, 3½ months of age and weighing 75 to 90 lb., came from a pig "hatchery" where there was no record in the herd history of swine erysipelas. These were crossbred Duroc, Poland China, and Chester Whites in excellent health. The third group of 20 shoats was purebred Chester Whites from a herd where no swine erysipelas has been observed since the herd was assembled in 1948. The remainder of the experimental animals were also purebred Chester Whites from a herd apparently free of swine erysipelas since it was established in 1941.

Three plans of management of the experimental animals were used. In plan A, the animals were kept in a barn in pens with cement floors. Self-feeders or feed troughs were used. In plan B, the animals were placed in a ¾-acre dry lot with self feeders. Small hog houses and shade were available. In plan C, the animals were placed on a 2-acre alfalfa and Ladino clover pasture with hog houses, shade, and self feeders. Hereafter, reference will be made to management plan A, B, or C.

The feed consisted of a well-balanced commercially prepared hog grower containing adequate minerals, vitamins, fat, and protein. When feed troughs were used the hogs were fed liberally twice daily.

Tryptose agar (Difco) was used for primary isolations of cultures. Later, stock cultures were incubated for twenty-four hours in a semisolid medium (Difco, brain, liver, heart—pH 7.2) plus 10 per cent horse serum and then placed in a refrigerator. Transfers were made at monthly intervals.

The identification of the organisms consisted of staining according to Gram's method, mouse and pigeon inoculations, and fermentation studies.

Routinely, each of 8 mice received 0.1 cc. of a 24-hour semisolid culture subcutaneously. Five of the group received 0.5 cc. of a potent swine erysipelas antiserum intraperitoneally. The antiserum was supplied by the B.A.I. of the U.S.D.A. Each pigeon used was given 0.5 cc. of the same culture injected into the pectoral muscles. Eight different cultures of *Ery. rhusiopathiae* were isolated from hogs with naturally occurring less acute swine erysipelas, one from a hog with advanced chronic erysipelas showing arthritis, and the other seven from hogs with acute septicemic erysipelas. Standard laboratory procedures for total white cell counts, differential white cell counts, and sedimentation rates were used for blood studies on normal and infected swine. All blood samples were drawn from the anterior vena cava.

RESULTS

Experiment 1, Classification of Cultures.—Eight tubes of each of 17 carbohydrates were used in fermentation studies for each culture. The mediums were sterilized by autoclaving at 15 lb. of pressure for fifteen minutes. All inoculations were made by using a loop of a 24-hour culture of the organisms grown in the semisolid medium. Readings were made at forty-eight hours, and again at ninety-six hours, to see if further changes occurred. No gas was produced in any case. The results of these studies (table 1) are in general accord with those of Karlson and Merchant.[11]

Eight groups of mice having 8 individuals each were injected subcutaneously with 0.1 cc. of a 24-hour growth of each of the eight cultures in a semisolid medium. Five of the 8 mice in each group received 0.5 cc. of a potent swine erysipelas antiserum intraperitoneally. Evidences of an acute septicemic disease were present in all 24 mice not protected by antiserum by the forty-eighth hour postinjection. At least 1 mouse in each group died by the sixtieth hour, and all were dead by seventy-two hours. *Erysipelothrix rhusiopathiae* was isolated from the heart blood and liver of each mouse that died. The mice receiving antiserum remained well.

An intramuscular injection of 0.5 cc. of a 24-hour growth of organisms from each of the eight cultures was made into 8 pigeons. All of the pigeons were visibly ill by forty-eight hours and all were dead by seventy-two hours. Cultures made from their heart blood, and liver were positive for *Ery. rhusiopathiae.*

Experiment 2, Repeated Exposure of Swine to Cultures of Erysipelothrix Rhusiopathiae Isolated from Swine with Chronic Arthritis and Cultivated on Tryptose Agar.—A rough colony of *Ery. rhusiopathiae* was isolated from the carpal and tarsal joints of a hog with naturally occurring chronic arthritis. Using the technique of Marsh,[18] the organisms were teased off the surface of a 48-hour growth in a tryptose agar tube into 3 cc. of physiological saline solution. All injections in this experiment were from this source of material.

a) Each of 5 hogs received 3 cc. of the suspension of organisms subcutaneously at weekly intervals for three weeks, was given three weeks' rest, and then each received 6 cc. subcutaneously. Two untreated hogs were placed in contact with them.

b) Each of 5 hogs received three weekly intramuscular injections of 3 cc. of the suspension of organisms. After a three-week interval each received 6 cc. of the suspension intramuscularly. Two hogs were placed in contact with them.

c) Each of 5 hogs received three weekly intra-

TABLE 1—Results of Fermentation of Carbohydrates

Carbohydrate	Culture Number							
	I	II	III	IV	V	VI	VII	VIII
D-glucose	+	+	+	+	+	+	+	+
D-fructose	±	+	±	±	±	+	+	±
D-galactose	±+*	+	±	+	+	+	+	+
D-mannose	−	±	−	−	−	±	−	±
Arabinose (l)	±	±	±	±	±	±	±	±
Xylose (d)	±	±+*	±	±	±	±	±	±
Lactose	−	±	±	±	±	±	±	±
Maltose	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−
Dextrin	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−
Soluble Starch	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−
Glycerol	−	−	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−	−	−

+ Acid; ± slight acid; − no acid; *—96 hour.

venous injections of 3 cc. of the suspension of organisms in the marginal ear vein. Three weeks later each received 6 cc. intravenously in the ear vein. Two hogs were placed in contact with them.

The experiment gave almost completely negative results. Only 2 hogs in group "*c*" (intravenous injections) showed a temperature of 150.0 F. and 105.5 F. for two days following the original injections. None of the cardinal symptoms of erysipelas, acute or chronic, were manifested. All animals remained healthy and made good weight gains during the experiment. Their previous herd history was not available except that they had been vaccinated against hog cholera at weaning time.

Experiment 3, Intravenous Exposure with a Smooth Colony of Erysipelothrix Rhusiopathiae Isolated from an Acute Septicemic Field Case.—It was decided to use a new approach to the problem by using shoats from herds known to be free from swine erysipelas, by using a smooth-type colony from a naturally occurring acute septicemic case, and by culturing the organism in a semisolid medium which has a reduced oxygen pressure and enriched with 10 per cent horse serum.

a) Each of 17 shoats received 10 cc. of a 24-hour culture of the organisms intravenously in the anterior vena cava. Twelve untreated shoats were placed in contact with these animals.

b) Each of 14 shoats received 20 cc. of a 24-hour culture of the organisms in the anterior vena cava. Four shoats were placed in contact with them.

In experiment "*a*," 6 of the 17 shoats injected intravenously died of acute swine erysipelas during the first six days and 5 others developed chronic erysipelas with arthritis. The other 6 shoats showed no febrile response or any other evidences of illness.

Fig. 1—Extensive erythema of the skin of a white pig in acute swine erysipelas four days following intravenous exposure to Erysipelothrix rhusiopathiae.

Fig. 2—Radiogram of the carpus of a hog nine months after exposure to Erysipelothrix rhusiopathiae, showing obliteration of joint spaces, ankylosis, and osteophyte formation. Metacarpal bones on the right.

At necropsy, the 6 shoats which died of acute erysipelas showed the usual gross pathological changes associated with acute septicemic swine erysipelas. Numerous bluish purple areas were present on the abdomen, across the sides, under the neck, and on the ears (fig. 1). The superficial lymph nodes were edematous, swollen, hemorrhagic and had a mottled appearance. The spleen was enlarged and had numerous deep bluish purple areas. A few small petechial subcapsular hemorrhages were present in the cortex of the kidneys. The liver appeared swollen, congested, soft, and friable. An extensive dark red hemorrhagic gastritis was observed in the lower half of the stomach and the inflammation of a lesser degree extended into the upper third of the duodenum. The gastrohepatic and mesenteric lymph nodes were hemorrhagic and edematous. The pancreas appeared normal except for the head which seemed firm and enlarged. A few epicardial hemorrhages with some congestion were observed on the heart. The adrenal glands were enlarged, soft and had many cortical hemorrhages. Cultures from the lymph nodes, head of the pancreas, brain, heart blood, liver, spleen, and adrenal glands were positive for *Ery. rhusiopathiae*.

Two of the 5 animals which developed chronic arthritis recovered spontaneously sixty days postexposure, while the other 3 developed marked enlargements of the

joints. One animal was unable to get up without assistance sixty days postexposure and was killed seven months later. At necropsy, extensive proliferative coxitis and spondylitis were present. The carpal and tarsal joints were enlarged, and bony ankylosis and exostosis had occurred as shown by radiograph (fig. 2). The left adrenal gland was rounded, flat and firm, suggesting some rearrangement of the normal architecture (fig. 3). The kidneys were enlarged and dull gray in color. Numerous scars, 20 to 30 mm. in diameter, resembling healed infarcts were present. All other viscera appeared to be normal. Bacteriological cultures made from the leg joints were negative.

The other 2 animals were killed six months postexposure and showed advanced polyarthritis and bony and fibrous enlargements of the carpal and tarsal joints. Cultures yielded rough colonies of *Ery. rhusiopathiae*.

One of the 12 contact animals died of acute swine erysipelas on the seventh day and *Ery. rhusiopathiae* was isolated from the liver, spleen, kidneys, adrenal glands, and heart blood. Three of the 12 developed chronic erysipelas with an arthritis which persisted. At necropsy, six months following exposure, marked fibrous and bony enlargements of the carpal and tarsal joints were present. The kidneys of each were enlarged, gray, and fibrotic. All other viscera appeared normal. Bacteriological cultures were positive for *Ery. rhusiopathiae*. The remaining 8 shoats resisted exposure.

In experiment "*b*," 12 of the 14 inoculated shoats died of acute swine erysipelas three to six days postexposure, 1 developed chronic arthritis, and the remaining animal resisted exposure. At necropsy, the usual gross pathological changes observed in acute swine erysipelas were present. Cultures made from the liver, kidneys, and spleen of all 12 animals were positive for *Ery. rhusiopathiae*.

The animal which developed chronic arthritis five months later had enlarged carpal and tarsal joints and a chronic proliferative coxitis. A marked proliferation of the synovial villi, pannus formation, and thickened synovial membranes and joint capsules were present. Cultures from the carpal and tarsal joints were positive for *Ery. rhusiopathiae*. All other tissues were normal in appearance.

Two of the 4 contact animals died of acute swine erysipelas on the eighth day following exposure. Their body temperatures were elevated at seventy-two hours, reached a peak at 120 hours, and the animals died two days later. Cultures were positive for *Ery. rhusiopathiae*. The other 2 shoats remained healthy.

Blood studies from 20 hogs from experiments "*a*" and "*b*" were selected for comparison. Daily white blood cell counts were used from each of 4 animals which died at forty-eight hours; 4 which died at seventy-two hours; 4 which died at ninety-six hours; 4 which died at 120 hours; and 4 which survived after being acutely ill and showing a high temperature. Daily white blood cell counts were also made on 4 normal animals and the results of all of these counts are given in figures 4 to 9. These figures also show body temperature, total white cell counts, and percentage of lymphocytes, monocytes, and neutrophils. The average count made on 4 normal shoats (graph 1) shows that the white cell counts per cubic millimeter of blood varied from 13,200 to 15,375 over a six-day period. The average percentage of distributions were:

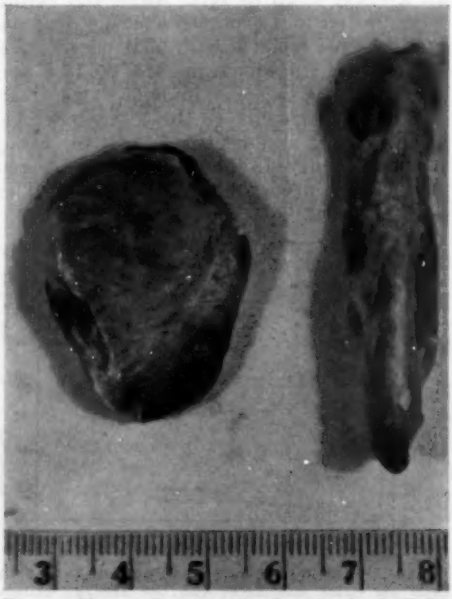
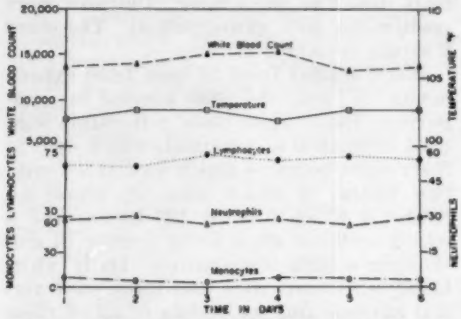


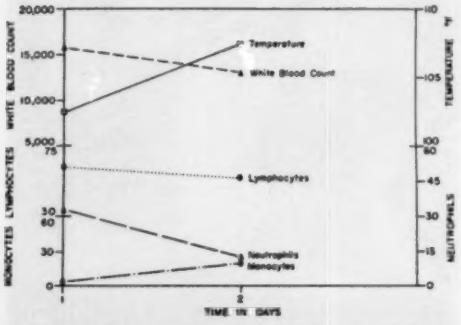
Fig. 3—Abnormally shaped adrenal gland (left) as compared to a normally shaped gland (right). Both were taken from a hog with chronic arthritis nine months postinoculation.

lymphocytes, 62.8 to 70.0; monocytes, 4.5 to 9.3; neutrophils, 26.8 to 31.0; eosinophils, 0.75 to 3.5; and basophils, 0.0 to 0.5 per cent. These results compare favorably with the counts reported by other investigators.



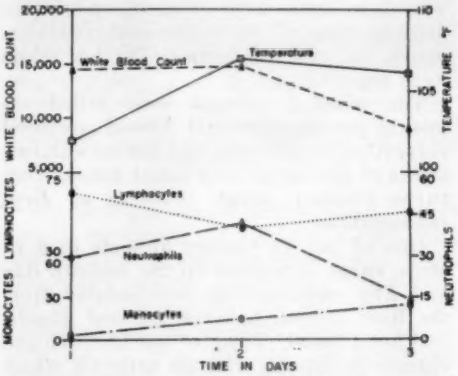
Graph 1—The average white blood cell count in cells per cubic millimeter of 4 normal swine for six days. The average differential counts are expressed as percentages.

Four pigs which were dead at forty-eight hours (graph 2) had an average temperature of 107.5 F. at twenty-four hours, an average drop in total white cell count from 15,785 to 13,850, an average drop in lymphocytes from 61.8 to 55.0 per cent, a drop in neutrophils from 33.0 to 12.5 per cent, and an increase in monocytes from 4.5 to 22.0 per cent. In the 4 shoats which were dead at seventy-two hours, the same pattern occurred; however, the changes were more pronounced (graph 3). The



Graph 2—The average white blood cell counts in cells per cubic millimeter and the average differential counts in percentages of 4 swine which died within forty-eight hours following intravenous exposure to Erysipelothrix rhusiopathiae.

average total white cell count dropped from 14,650 to 9,238, lymphocytes from 63.8 to 53.8 per cent, and neutrophils from 30.0 to 14.3 per cent. The average temperature rose from 102.0 F. to 107.0 F. in twenty-four hours and was 106.1 F. at forty-eight hours. The monocytes rose from 2.5 to 25.8 per cent. The group of shoats which died at ninety-six hours showed a similar response in the white cell counts (graph 4). The temperature rose from 102.0 F. to 107.0 F. in twenty-four

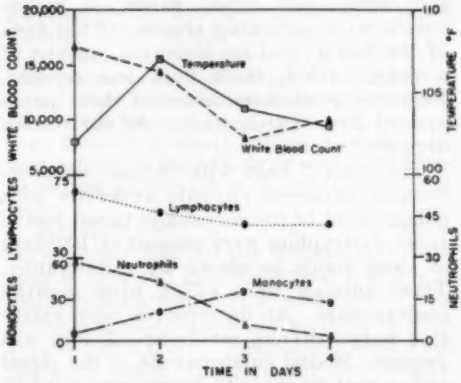


Graph 3—The average white blood cell count in cells per cubic millimeter and the average differential counts in percentages of 4 swine which died within seventy-two hours following the intravenous exposure to Erysipelothrix rhusiopathiae.

hours, was 104.0 F. at forty-eight hours and 103.0 F. at seventy-two hours. The average total white cell count dropped from 16,687 to 14,382 at twenty-four hours, 8,387 at forty-eight hours and was 10,000 at seventy-two hours following exposure. The lymphocytes dropped from 66.5 to 55.0 per cent at twenty-four hours, 47.5 per cent at forty-eight hours, and were 48.0 per cent at seventy-two hours. The neutrophils dropped from an average of 28.8 to 22.3 at twenty-four hours, 6.3 at forty-eight hours, and 1.8 at seventy-two hours postinjection. The most pronounced change in white blood cell counts occurred in the shoats which died at 120 hours postexposure (graph 5). The body temperature rose from 102.0 F. to 106.1 F. in forty-eight hours and dropped to 101.8 F. at ninety-six hours. The average white cell count dropped about two-thirds, from 19,175 to 6,287, at seventy-two hours then rose to

12,412 at ninety-six hours. The lymphocytes dropped from 53.3 to 32.3 per cent in seventy-two hours, a 40.0 per cent drop. The neutrophils dropped from an average of 41.8 per cent to 0.0 at seventy-two hours. The monocytes rose from an average of 3.8 per cent to 53.8 at seventy-two hours, a fourteenfold increase.

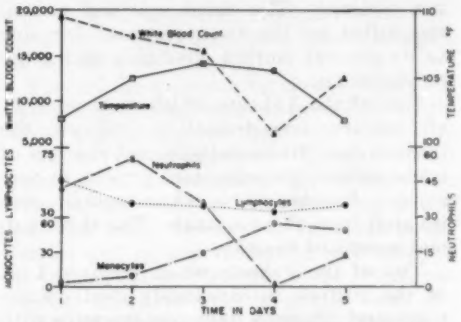
In the group of shoats which showed an acute febrile response but recovered, the maximum white blood cell changes occurred at forty-eight hours postexposure



Graph 4—The average white cell count in cells per cubic millimeter and average differential counts in percentages of 4 swine which died by the ninety-sixth hour following intravenous exposure to Erysipelothrix rhusiopathiae.

(graph 6). The body temperature rose from 102.5 F. to 106.8 F. in forty-eight hours and then dropped to 103.1 F. at 120 hours. The average total white cell count per cubic millimeter of blood dropped from 14,000 to 11,037 at forty-eight hours and rose to 16,337 at 144 hours. The lymphocyte counts dropped from an average of 69.3 per cent to 54.0 at forty-eight hours and rose to 60.0 per cent by 144 hours. The average neutrophil count dropped from 26.8 to 14.0 per cent at forty-eight hours and rose to 28.3 at 144 hours. The average monocyte count rose from 4.3 to 31.5 per cent at forty-eight hours and then dropped to 7.8 at 144 hours. The same general pattern of white cell response was observed in this group as in the previous groups which died, but it was less pronounced. In these animals, the maximum changes occurred at forty-eight hours postexposure and then a gradual return to normal in body temperature and white cell count had occurred by 144 hours postexposure.

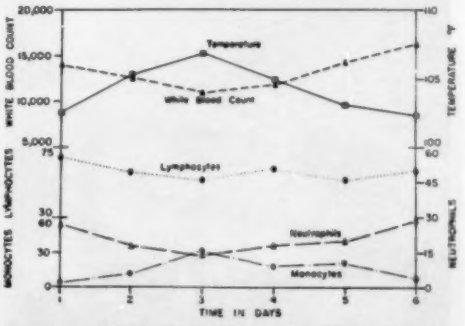
Experiment 4, Production of Swine Erysipelas and Arthritis in Shoats Not Vaccinated for Hog Cholera.—This experiment was designed to observe the effects of *Ery.*



Graph 5—The average white cell count in cells per cubic millimeter and average differential counts in percentages of 4 swine which died by the 120th hour following intravenous exposure to Erysipelothrix rhusiopathiae.

rhusiopathiae upon shoats not vaccinated for hog cholera since all the other shoats used in the experiment had been vaccinated against hog cholera by the serum and virus method at weaning time (8 to 10 weeks of age).

The 14 shoats used were 3½ months of age and weighed from 75 to 90 lb. Management plan A was used. Each of 3 shoats received 1 cc. of a 24-hour culture intradermally; each of 3 shoats received 1 cc. of culture intravenously; and each of 3 shoats



Graph 6—The average white cell count in cells per cubic millimeter and average differential counts in percentages of 4 swine which survived acute illness following intravenous exposure to Erysipelothrix rhusiopathiae.

were scarified superficially and 2 cc. of the culture applied topically. Five other shoats were placed in contact with these animals.

Two of the scarified shoats died of acute swine erysipelas on the seventh and eighth days postexposure. At necropsy, typical lesions were present and *Ery. rhusiopathiae* was isolated. The third scarified animal was killed on the twenty-second day due to its general morbid condition and acute polyarthritis.

Two of the 3 shoats which received 1 cc. of culture intradermally died on the twelfth day. Gross pathological changes of acute swine erysipelas were present at necropsy. *Erysipelothrix rhusiopathiae* was isolated from these animals. The third animal remained healthy.

Two of the 3 shoats which received 1 cc. of the culture intravenously died on the tenth and fifteenth days postexposure with lesions typical of acute swine erysipelas. Cultures were positive for *Ery. rhusiopathiae*. The third shoat remained healhty.

Two of the 5 contact shoats died on the fourteenth day. Gross lesions of acute swine erysipelas were present at necropsy, and cultures were positive for *Ery. rhusiopathiae*. The other 3 shoats remained healthy.

Experiment 5, Production of Swine Erysipelas and Arthritis by Exposure Through Skin Scarification.—In this experiment, 19 shoats from a pig "hatchery" were used. Of these, 13 were scarified on the side, and 2 cc. of a 24-hour culture of the organisms was applied on the scarified area. The other 6 shoats were scarified and placed in contact with these animals.

Fig. 4—Fibrous ankylosis of the carpal joint of a shoat 135 days after exposure to Erysipelothrix rhusiopathiae. x 100.

Three of the 13 inoculated shoats died of acute swine erysipelas by the eighth day and 4 others developed erysipelas with chronic arthritis. All showed well-marked local reactions but 6 remained healthy. Two of the chronically affected were killed four months postexposure because of their morbid and weakened condition. At necropsy, there was marked synovitis with proliferation of the synovial villi. Small erosions were present on the articular cartilages of the carpal and tarsal joints. A severe coxitis with beginning erosion of the neck of the femur, and an increased amount of a flaky, turbid, thick fluid was present. Bacteriological examination of these joints yielded *Ery. rhusiopathiae*. All the viscera appeared normal.

The other 2 hogs with chronic cases developed advanced chronic arthritis with enlargement of the carpal and tarsal joints. Bony dystrophies were present at 100 days in these joints as shown by radiography. These animals were killed nine months postexposure. At necropsy, a very extensive polyarthritis involving all legs was present. Medial enlargements of the tarsal and carpal joints with bony ankylosis had occurred. Pannus formation and numerous erosions of the articular cartilages were present. The joint capsules were thickened and marked proliferation of the synovial villi was present. Bacteriological examination of these joints gave negative results. In 1 shoat, the kidneys were gray and fibrotic. The combined weight of the adrenal glands was 7.450 Gm. and each had two small cortical cystlike structures the size of a pea. All other viscera appeared normal. In the other animal, the kidneys were gray and had many scars 20 to 30 mm. in diameter in the cortex. Much fibrosis had developed. The adrenal glands weighed 8.070 Gm. and appeared normal.

Two of the 6 scarified contact shoats died of acute swine erysipelas by the eighth day and showed gross lesions of this disease. Bacteriological cultures from the liver, spleen, and kidneys were positive for *Ery. rhusiopathiae*.

One contact shoat developed chronic arthritis. At 100 days postexposure, the carpal and tarsal joints were enlarged. Radiographs revealed osteophyte forma-

tions and narrowing of the joint spaces. The arthritic condition became worse and the animal was killed 135 days postexposure. The carpal and tarsal joints were devoid of synovial fluid and fibrous adhesions were beginning to form between the articular surfaces immobilizing the joints (fig. 4). The joints yielded *Ery. rhusiopathiae*. The viscera appeared normal. All of the contact animals showed local reactions but 3 remained healthy.

Experiment 6, Production of Swine Erysipelas and Arthritis by Intra-articular Exposure.—In these experiments, 49 of the Chester White shoats were used.

a) Each of 14 shoats received 1 cc. of a 24-hour culture in the left carpal and right tarsal joints. Eight noninoculated shoats were placed in contact with them.

b) Each of 15 shoats received 2 cc. of a 24-hour culture in the left carpal and right tarsal joints, and 12 shoats were placed in contact with them.

In experiment "*a*," 3 of the 14 inoculated shoats died on the seventh and fourteenth days and showed typical features of acute swine erysipelas; *Erysipelothrix rhusiopathiae* was isolated from their spleens, livers, and kidneys. Four others died while showing acute arthritis on the twenty-fourth, thirty-second, thirty-fifth, and fifty-third days, respectively. Marked distention of both carpal and both tarsal joints with a thick, turbid, serosanguineous fluid was present at necropsy. No difference could be detected between the injected joints and the corresponding joints in the opposite legs. The synovial membranes were inflamed and marked proliferation of the villi had occurred. Cultures made from all carpal and tarsal joints were positive for *Ery. rhusiopathiae*.

One shoat died 112 days after exposure and showed extensive vegetative endocarditis (fig. 5) and numerous healed infarcts in the kidneys (fig. 6).

Five of the 6 remaining shoats developed chronic erysipelas with advanced arthritis which persisted until they were killed 120 to 421 days after exposure. Extensive bony dystrophies, as revealed by radiography, were present. Marked proliferation of the synovial villi, thickened synovial membranes, and pannus formation had occurred in the carpal and tarsal joints. Bacteriological cultures were negative from the joints of all of these animals. The sixth animal recovered spontaneously after 180 days.

Three of the 8 contact animals died by the ninth day of generalized swine erysipelas. At necropsy, the gross pathological changes were typical of acute swine erysipelas. Cultures made from the spleens, livers, and kidneys were positive for *Ery. rhusiopathiae*. Two others developed advanced chronic arthritis and spondylitis (fig. 7). Bacteriological cultures of the carpal and tarsal joints were negative at necropsy (240 and 330 days). Only 3 of the contact shoats remained healthy.

Fig. 5—Extensive vegetative endocarditis in a shoat 112 days after intra-articular inoculation of Erysipelothrix rhusiopathiae culture. This animal also had chronic arthritis.

In experiment "*b*," no deaths occurred from acute swine erysipelas in the 15 inoculated shoats. Five were ill but 3 recovered spontaneously at sixty days and 2 at

Fig. 6—Infarcts in the kidney of a shoat with chronic arthritis 112 days after intra-articular inoculation with Erysipelothrix rhusiopathiae.

120 days. While 4 remained healthy, 6 others developed polyarthritis. One of these had acute polyarthritis, became moribund at fifty-four days and was killed. Both carpal and tarsal joints were swollen and distended with a serosanguineous fluid. Marked inflammation of the joints had occurred with proliferation of the villi. Cultures were positive for *Ery. rhusiopathiae*. Numerous scars were present in the cortex of the kidneys, while all other viscera appeared normal.

One of the 6 was killed 226 days after exposure because of severe dystrophy of one hind leg. At necropsy, the carpal and tarsal joints were enlarged medially with osteophytic formations, as shown by radiography. On the medial aspect of the neck

Fig. 7—Extensive new bony growths on the articular processes of the lumbar vertebrae of a hog with chronic arthritis nine months after contact exposure to Erysipelothrix rhusiopathiae.

of the femur, there was an osseous mass 7 to 8 cm. in diameter. Removal of this tissue revealed an erosion of the neck of the femur. Extensive pannus formation had occurred in the carpal and tarsal joints with extensive proliferation of the synovial villi. Cultures from the joints were positive for *Ery. rhusiopathiae*. The articular processes of the lumbar vertebrae were found to be enlarged and ankylosed. The kidneys were gray and fibrotic. All other viscera appeared normal. The 4 other hogs with chronic cases were similar in gross appearance to those previously described as advanced arthritis cases.

One animal in this group, killed at 120 days, had a subcutaneous nodule 20 by 30 mm. attached to the superficial digital flexor tendon. It had a thick fibrous capsule and the center was filled with a sterile, serosanguineous fluid. This nodule apparently resulted from trauma. *Erysipelothrix rhusiopathiae* was isolated from the carpal and tarsal joints. Bacteriological examination of the joints of the other 3 with chronic cases, which were killed between 188 and 356 days, gave negative results.

Two of the 12 contact animals died on the fifteenth and sixteenth days, respectively, of acute swine erysipelas. Cultures of the spleen, liver, and kidneys were positive for *Ery. rhusiopathiae*. One shoat developed chronic polyarthritis, became moribund, and was killed 189 days postexposure. Gross pathological changes typical of chronic erysipelas arthritis were present at necropsy, and cultures of the leg joints were positive for *Ery. rhusiopathiae*. Another shoat developed chronic arthritis and was killed at 328 days. A severe coxitis was present with an accumulation of fluid of a flaky, turbid, and viscous nature. Radiography revealed osteophytic formations in the region of the tarsal and carpal joints. Cultures from these joints were negative. The 8 other contact animals remained healthy.

Experiment 7, Production of Swine Erysipelas and Arthritis on Alfalfa Pasture.—This experiment was designed to study contact transmission under field conditions and, also, to compare the rate of arthritic changes with that which occurred in swine kept more closely confined. Thirty Chester White shoats were placed on a 2-acre pasture of alfalfa and Ladino clover.

Each of 6 shoats received 10 cc. of a 24-hour culture of *Ery. rhusiopathiae* intramuscularly; 6 received 10 cc. subcutaneously; and 6 received 2 cc. each in the right tarsal and left carpal joints. Twelve shoats were placed in contact with these animals. Three of the 6 animals which were injected intramuscularly developed polyarthritis and were killed on the 203rd, 204th, and 313th days. Gross pathological changes including bony dystrophies were present. All viscera appeared normal. *Erysipelothrix rhusiopathiae* was isolated from the carpal and tarsal joints of the first 2 but not from the third shoat. The other 3 shoats resisted exposure.

Two of the 6 animals which were injected subcutaneously died on the fifth and tenth days, respectively, of acute swine erysipelas. Gross pathological findings were typical and *Ery. rhusiopathiae* was isolated from the spleens, livers, and kidneys. One of the 6 developed advanced chronic polyarthritis and the other 3 remained healthy.

Two of the 6 shoats injected intra-articularly died of acute swine erysipelas. The organism was isolated from the livers, spleens, and kidneys of these animals. One shoat developed advanced chronic polyarthritis and the other 3 remained healthy.

Three of the 12 contact shoats died of acute swine erysipelas on the eighth, eleventh, and fourteenth days. The organism was isolated from the spleens, livers, and kidneys of these animals. One hog with a chronic case died on the 124th day with extensive vegetative endocarditis involving the bicuspid valve and chordae tendineae. It also showed proliferative coxitis, carpitis, and arthritis in the tarsus. Cultures from the vegetative masses of the heart, the carpal, and tarsal joints were positive for *Ery. rhusiopathiae*. Eight of the 12 contact shoats remained healthy.

Experiment 8, Observations on Pathological Changes in Acute and Chronic Swine Erysipelas.—The lesions of swine erysipelas are varied. In animals which die of the acute septicemic form of the disease, pathological changes observed at necropsy may be no different from those observed in any other septicemic disease. The skin lesions perhaps are more nearly pathognomonic than any other finding.

Damage to the blood vessels of the skin, resulting in the peculiar type of erythema, presumably was due to the action of the organism and its by-products. The histopathological changes occurred principally in the papillae, subpapillary, and upper layers of the dermis. The blood vessels were distended, the papillae were enlarged and showed marked hyperemia. Frequently, small hemorrhages were observed in the connective tissue elements of the papillae (fig. 8). Numerous foci of round cells and necrotic areas were present.

In the urticarial lesions, a marked edema of the skin occurred in the wheals. Numerous thrombi and dilation of the vessels were present. Capillary hemorrhages appeared especially in the papillary layer. The chief difference in the skin lesions of animals with the acute septicemic form and those showing urticaria chiefly was in the degree of damage done. The greater

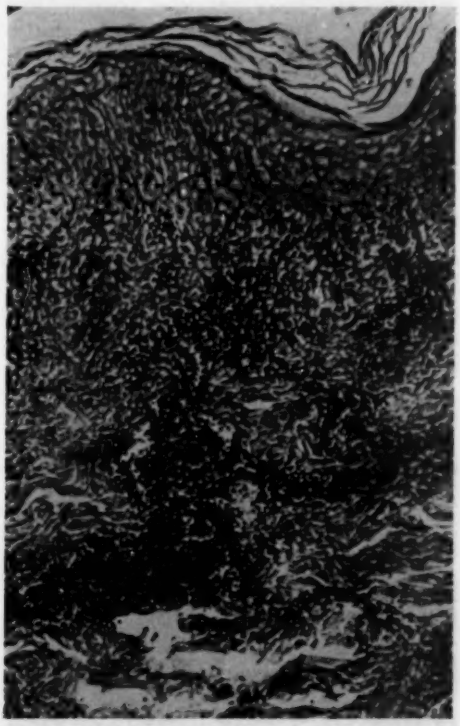


Fig. 8—Hemorrhages in the skin of a hog with an experimentally produced case of acute swine erysipelas. x 200.

damage is likely to occur in the septicemic form in which local areas may become gangrenous if the animal survives. Much hemorrhage and engorgement occurred in the subcutaneous fat in these areas.

The skeletal muscle ordinarily did not show degenerative changes. Vascular dilation with engorgement may occur in any tissue, however. In acute cases in which there was involvement of the heart, the myocardium frequently contained numerous hemorrhages and cloudy swellings. Sometimes ecchymoses were observed under the endocardium. The serous membranes ordinarily were not affected to any great extent. Sometimes fibrinous exudate was found on the serous surfaces and fibrous changes occurred resulting in firm adhesions between serous surfaces. A serofibrinous pericarditis sometimes occurred.

The lymph nodes usually had many hemorrhages and showed depletion of the germinal centers (fig. 9). There also was evidence of edema. Marked eosinophilic infiltration of lymph nodes was frequently found in chronic cases. The spleen was moderately enlarged and numerous hemorrhages were frequently present.

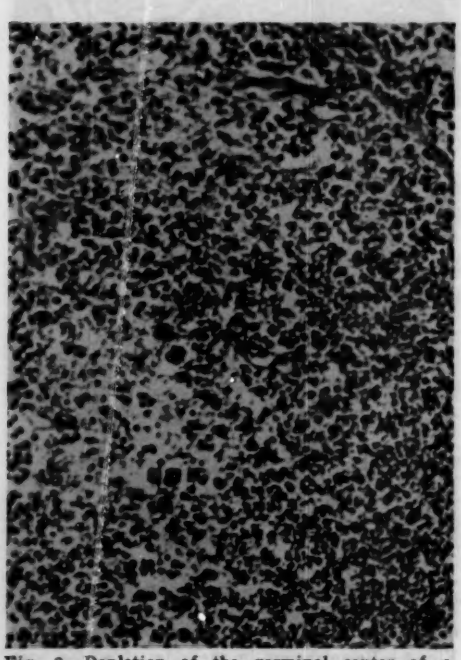


Fig. 9—Depletion of the germinal center of a lymph node from a hog with a case of experimentally produced acute swine erysipelas. x 200.

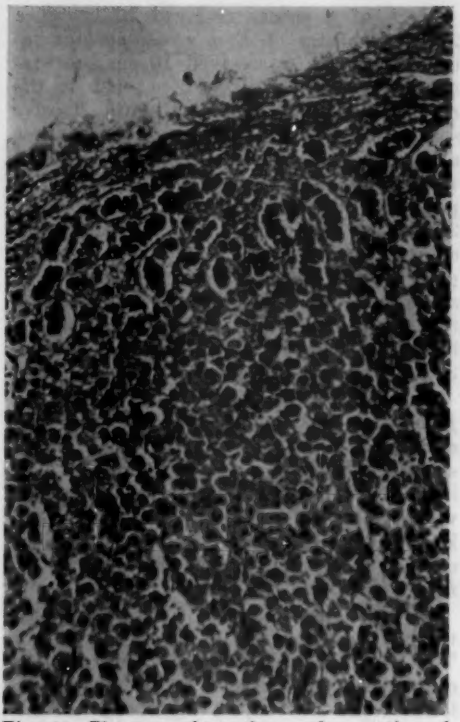


Fig. 10—The zona glomerulosa and a portion of the zona fasciculata of the adrenal gland from a normal adult hog. x 200.

In the digestive tract, pathological changes varied from a severe inflammation of the lower half of the stomach and the upper third of the duodenum, to no involvement. The longer the animal survived during the acute phase of the disease, the greater the incidence of gastroenteritis. In severe cases the mucosa was a dark red, the submucosa was swollen and infiltrated with a serosanguineous fluid. The epithelium of the area was usually more or less destroyed and the glands degenerated.

The liver was enlarged and engorged, the sinusoidal spaces distended with blood, and numerous small hemorrhages were present, particularly near the center of the lobules. In the acute phase of the disease, the pathological change was essentially an extensive engorgement.

In the kidneys, subcapsular hemorrhages were sometimes present, depending upon

the acuteness of the disease. There were evidences of acute inflammation, degenerative changes in the parenchyma, and interstitial hemorrhage. Infarcts were occasionally necrotic or, in prolonged cases, they may have been replaced by connective tissue and eventually undergone cicatrization.

In acute swine erysipelas, the adrenal glands were enlarged and frequently subcapsular hemorrhages were observed. In chronic cases of two months' or more duration, the adrenal glands were frequently twice normal size, very soft and swollen; the zona glomerulosa infiltrated with many leukocytes, and complete or almost complete disappearance of this zone occurred in some cases (fig. 10 and 11). Numerous foci of round cells were often scattered throughout the fascicular and reticular zones. It is not certain that these foci resulted from *Ery. rhusiopathiae* infection. The medulla usually appeared normal but some showed atrophic or degenerative changes. In advanced cases of chronic erysipelas arthritis, a disruption of the normal architecture of the glomerulosa and reduction in thickness occurred.

In the acute stage of arthritis, the principal changes were characterized by vascular engorgement of the joint capsule and synovial tissues and more or less edema. The effusions were turbid or serosanguineous or mucinous. The synovial villi showed beginning proliferation, extensive vasodilation, engorgement, and beginning lymphocytic infiltration (fig. 12 and 13).

In arthritis of two months' duration, greater proliferation and less edema was observed. Much proliferation of the cells covering the hypertrophied synovial villi was present. The villi contained young, highly vascular connective tissue, infiltrated with plasma cells and lymphocytes. A striking feature was the dense collection of lymphocytes. The villi of arthritic joints of six months' or longer duration frequently resembled granulomatous polyps (fig. 14). Very few polymorphonuclear leukocytes were observed and the infiltrating cells were principally lymphocytes, plasma cells, and eosinophils. Relatively few areas of suppuration were observed as described by Collins and Goldie.[5] Less extensive destruction of cartilage and bone than that which characterizes suppurative arthritis in other species was observed. The synovial effusions were nonpurulent and contained mostly monocytes, lymphocytes, and a few synovial lining cells.

In advanced chronic arthritis with pannus formation of subchondral origin, marked fibrosis of the adjacent marrow spaces, increased vascularity, and collections of lymphocytes occurred. In those cases in which the pannus apparently originated from the synovial membrane, subchondral fibrosis was less likely to occur. Pannus attachment to the articular cartilage was accompanied by erosions and destruction of the cartilage. In advanced cases with little or no synovial fluid, intra-articular fibrous adhesions formed, apparently in an attempt to immobilize the joint.

Radiograms revealed osteophyte formation with apparent ankylosis of the carpal and tarsal joints. Also, the articular processes of the vertebrae frequently appeared to be fused. Irregular ossification and rare-

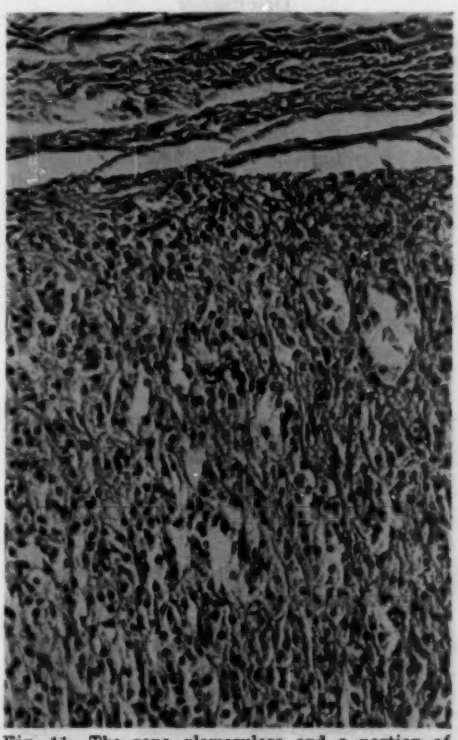


Fig. 11—The zona glomerulosa and a portion of the zona fasciculata of the adrenal gland from a hog which had experimentally produced chronic arthritis. The zona glomerulosa is nearly obliterated. The fascicular zone shows atrophy of the parenchymatous cells and numerous spaces. The capsule appears edematous. x 200.

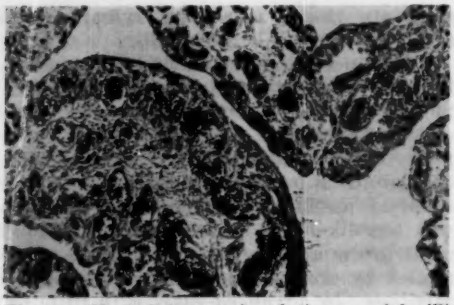


Fig. 12—Marked hyperemia of the synovial villi in a hog with experimentally produced acute erysipelas arthritis. x 100.

faction of the bones adjacent to the joints were frequently observed. In hogs which spontaneously recovered, no gross abnormalities were observed.

DISCUSSION

The difficulty experienced by many investigators in attempting to reproduce swine erysipelas in hogs has caused them to use the mouse and pigeon for the identification and classification of the organism. The pigeon and mouse succumb in two to three days when injected with small quantities of a 24-hour broth culture. They may be protected by the simultaneous administration of potent swine erypsipelas antiserum.

The fermentation of carbohydrates is a less reliable criterion for classification and identification of *Ery. rhusiopathiae* than morphological and tinctorial features, combined with mouse and pigeon inoculations. The variation in ability of the organism to ferment carbohydrates in different laboratories has been reported. No doubt strain differences, age of laboratory strains, mediums used to cultivate the stock cultures while in storage, and the addition of animal proteins to the carbohydrates for fermentation studies have all played a part in the variable results obtained. It appears that there is a relatively small group of closely related carbohydrates which may be fermented by *Ery. rhusiopathiae,* in which differences in the ability of certain cultures to ferment them do exist. Even so, when allowance is made for these known differences, and the morphological and tinctorial properties are combined with the mouse and pigeon inoculations in protected and unprotected individuals, the organism can be identified.

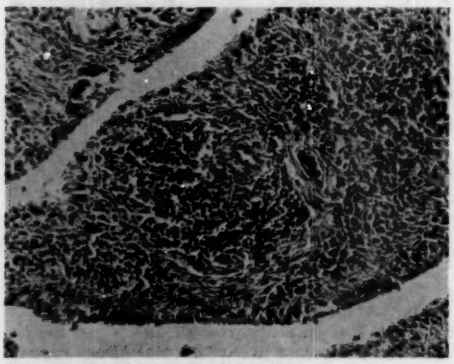


Fig. 13—Beginning proliferation of the synovial villi with focal round cell accumulation in a hog with experimentally produced erysipelas arthritis. x 100.

The organism is micro-aerophilic, and may revert to rough colony formation when grown at ordinary atmospheric oxygen pressure on tryptose agar. The organism may even become Gram negative when cultivated for long periods on tryptose agar. Even in the advanced chronic cases of naturally occurring erysipelas arthritis referred to in experiment 2 in which repeated exposure was used, a rough-type colony was isolated. This suggests the ability of the organism to adapt itself to a state of compatability with the animal body.

Three possible explanations may be given for the negative results obtained in experiment 2 in which the animals received repeated exposures. First, the shoats could have been exposed previously to the organism with a subclinical infection, since the complete health history was not available. Secondly, the organisms produced a rough-type colony suggesting low pathogenicity. Thirdly, the organism was cultivated in ordinary atmosphere on tryptose agar and suspended in physiological saline. Stickdorn [33] has shown that physiological saline may reduce the pathogenicity of the organism for mice. Thus, at least three possible contributing factors could have played a part in the negative results obtained in this experiment. These negative results are typical of the findings reported by many investigators.

Careful consideration of these three factors produced positive results in experiment 3. These factors were: (1) use of

shoats 3½ to 4 months of age from herds known to be free of swine erysipelas; (2) use of a semisolid medium which was enriched with 10 per cent horse serum; (3) the use of a smooth-type colony isolated from an acute septicemic case.

In experiment 4, the shoats which had not been vaccinated against hog cholera proved to be highly susceptible to *Ery. rhusiopathiae*, indicating that hog cholera vaccination with serum and virus was not a factor in the susceptibility of the other experimental animals. It would seem that other factors such as pathogenicity of the organism, resistance of the animal due to previous contact with the organism, state of nutrition and, perhaps, age, are the important variables in determining the animal's susceptibility to the infection.

From the results of the white blood cell studies which show a marked leukopenia, lymphopenia, neutropenia, and a monocytosis, it appears that the most critical period is from thirty-six to seventy-two hours following intravenous exposure. In such cases, therapy to fortify or aid the natural defenses should be given within twenty-four to thirty-six hours postexposure.

In experiment 5, in which the shoats were scarified and *Ery. rhusiopathiae* was applied topically, 3 of 13 (23 %) died of acute swine erysipelas. Four others (31 %) developed chronic erysipelas arthritis, while the remaining 6 (46 %) resisted exposure.

One animal with advanced chronic arthritis (9 months) had gray, fibrotic kidneys and renal function was undoubtedly impaired. The cystic condition of the adrenal glands suggests a possible interference with the normal production of cortical hormones. It is not certain that these kidney and adrenal changes played a role in the continued active arthritic condition.

The disadvantage of the scarification technique, when employed to determine the resistance of an animal, is that it may not insure adequate exposure. Probably, many of the organisms are rubbed off by the animals. Presumably, infection is dependent upon the organism entering the lymphatic system and establishing infection in the regional lymph nodes and eventually entering the blood stream. With the probability of a reduced number of the organisms gaining entrance into the lymphatic glands, skin scarification may fail to give sufficient challenge to the resistance of an animal.

In support of the above reasoning 8 of 29 (28 %) of the animals in experiment 6, which were injected intra-articularly, died of erysipelas as compared to 23 per cent which were scarified. Of the scarified animals 31 per cent developed arthritis, as compared to 51.8 per cent of those injected intra-articularly. Also, 46 per cent of the scarified animals resisted the exposure as compared to 17.2 per cent in the group injected intra-articularly. Thus, it seems that intra-articular injection would provide a more adequate exposure than skin scarification for a more accurate evaluation of the protective value of vaccinational methods when used on swine.

The clinical manifestations of the disease vary with the susceptibility of the animal. In the acute septicemic form, the animal

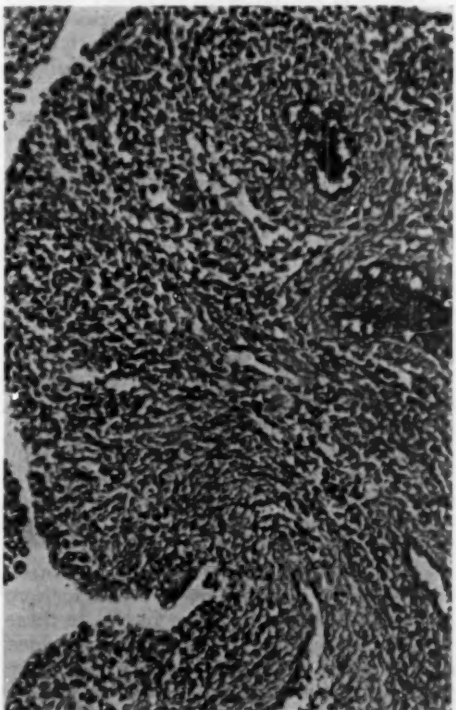


Fig. 14—A synovial villus from a hog with advanced chronic erysipelas arthritis, showing some granulomatous changes in addition to lymphoid cell infiltrations. x 200.

usually develops a temperature of 106 to 108 F., soon becomes listless, and goes off feed. If the fever remains high for twenty-four hours, respiration becomes rapid and shallow. Numerous reddish or purplish areas may appear over the body. In these animals, a marked leukopenia with a monocytosis occurs. If the fever subsides in a few hours, the animal may live. If, however, the fever remains very high for thirty-six to forty-eight hours, the animals become prostrate and death is very likely to occur.

Extensive damage to the small blood vessels occurs allowing blood to escape into the tissues. The loss of serum proteins and dehydration of the body apparently hasten death. In animals that survive, there are usually symptoms suggesting muscular soreness and tender feet or legs. Definite symptoms of arthritis are likely to develop later.

The skin lesions may be rhomboidal in outline or diffuse. Wheals form which may at first appear blanched but soon become erythematous and edematous. These wheals may subside after thirty-six to forty-eight hours or they may coalesce and form large sloughs. In either case, chronic joint involvement usually results, and the animal becomes lame. The blood cell changes are not as pronounced in these animals as in those with acute cases.

In hogs with chronic cases, the heart may be affected. At least some of these animals are survivors of an acute, septicemic infection. In experimental cases, the animals seem to recover from the acute phase of the disease but often remain stunted. At necropsy, extensive vegetative endocarditis involving either the tricuspid, or the bicuspid, valve is observed. The vegetations may be so large as to occlude the aperture. *Erysipelothrix rhusiopathiae* can often be isolated from these vegetative masses.

Chronically arthritic swine are often survivors of a previous acute infection. An actue arthritis develops in two to three weeks postexposure with effusions which distend the joint capsule. The animals are very lame and frequently shift the weight from leg to leg while standing. The appetite may be poor and a febrile response is usually not detectable *per rectum*. However, the swollen joints are hot, painful, and sensitive to the touch. In two or three weeks the acute swelling is reduced, but the lameness usually persists and, in many animals, one limb may not be used to bear weight. If the infection persists for two months or longer, the chance of recovery is slight. A marked periostitis, ostitis or, in rare severe cases, osteomyelitis is present. Extensive thickening of the joint capsule and osteophytic formations with ankylosis may develop. The authors were unable to isolate *Ery. rhusiopathiae* from the joints of most animals five months following exposure, but the pathological processes persist with periods of exacerbation and remission. These periods vary considerably and may be two weeks or more in length. An occasional spontaneous recovery occurs after several months, but the joints remain enlarged.

Collins and Goldie [5] believe that one of three things may happen in chronic arthritis due to *Ery. rhusiopathiae* infections: (1) the lesion may resolve and the joint recover; (2) healing of the damaged tissues may take place by fibrosis and this may include the formation of intra-articular adhesions; or (3) the synovial granulation tissue may assume certain properties of a benign neoplasm and persist and extend in the absence of the exciting microorganisms, thus maintaining the arthritis in a state of clinical activity. The results of the experiments reported in this paper support this latter belief, especially in the case of the continued active clinical form after seven months. Perhaps damage to adrenal glands and kidneys are important contributing factors in the continued active clinical manifestation of arthritis in swine.

SUMMARY

1) *Erysipelothrix rhusiopathiae* was isolated from 15 hogs with naturally occurring cases of swine erysipelas and from 1 with an advanced case of chronic arthritis.

2) Identification of the organism was based on staining according to Gram's method, mouse injection and protection tests with swine erysipelas antiserum and pigeon inoculation tests. Also, carbohydrate fermentation tests were made.

3) Repeated exposure of 15 hogs with an indefinite history using a rough-type culture gave almost completely negative results.

4) Intravenous exposure of unexposed shoats with 10 cc. of a 24-hour growth of a smooth-type culture isolated from a hog with an acute septicemic case and grown in a semisolid medium containing 10 per cent horse serum gave positive results. Six of 17 inoculated shoats died of acute swine erysipelas and 5 developed chronic arthritis. One of 12 shoats exposed by contact died of acute swine erysipelas and 3 developed chronic arthritis.

5) Twelve of 14 shoats which received 20 cc. of a 24-hour culture from the original smooth culture intravenously died of acute swine erysipelas. Two of 4 contact shoats also died of acute swine erysipelas.

6) Daily white blood cell counts revealed a marked leukopenia, lymphopenia, neutropenia, and a monocytosis within twenty-four to seventy-two hours post-exposure.

7) Shoats not vaccinated against hog cholera were susceptible to *Ery. rhusiopathiae* when exposed intradermally, intravenously, and by scarification. Six of 9 animals so exposed and 2 of 5 contact animals died of acute swine erysipelas.

8) Shoats vaccinated against hog cholera with the serum and virus method were no more susceptible than unvaccinated animals. Three of 13 vaccinated shoats which had the organisms applied topically on the scarified areas died of acute swine erysipelas and 4 others developed chronic arthritis, while 6 resisted infection. Two of the 6 vaccinated, scarified, contact control shoats died of acute swine erysipelas and 1 other developed chronic arthritis, while 3 remained healthy.

9) Intra-articular inoculation proved to be a highly effective route of exposure. Eighty-two per cent of the shoats died of acute swine erysipelas or developed advanced chronic arthritis.

10) A high incidence of acute and chronic swine erysipelas occurred in experimentally exposed animals on an alfalfa pasture. Contact exposure resulted in development of acute swine erysipelas and death in 3 of 12 shoats and a fourth died later showing vegetative endocarditis and chronic arthritis.

11) *Erysipelothrix rhusiopathiae* could not be isolated from animals with advanced chronic arthritis more than 226 days after exposure.

12) A discussion of the nature, course, and pathology of *Ery. rhusiopathiae* infections is given.

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Studies on Arthritis in Swine. II. The Effects of Hormonal Therapy on Advanced Chronic Polyarthritis Experimentally Induced by Erysipelothrix Infections

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THE ADRENAL hormones produce a number of profound physiological effects in the body.

The work of Britton and Silvette,[5] Britton and Corey,[6] Long,[11] Marrazzi,[12] and Russell [13] have shown that absorption, storage, formation, and utilization of carbohydrates are impaired by ablation of the adrenal cortices. These functions can be restored by the administration of the appropriate cortical hormones. Koepf *et al.*[9] and Kuizenga *et al.*[10] have shown that in fasting animals which were adrenalectomized, the excretion of nonprotein nitrogen is decreased, indicating that insufficient endogenous protein is being broken down in order to form carbohydrate.

It is known that the unfractionated cortical extract or the related steroids which are oxygenated at the eleventh carbon atom elevate the blood glucose and liver glycogen of normal and adrenalectomized animals even when fasted. This indicates that the carbohydrate is formed from some noncarbohydrate precursor. Also, an increase in excretion of nitrogen occurs principally at the expense of tissue protein. Some believe that a depletion of lymphatic tissue is the principal source of this nitrogen in the early stages in animals so treated. The group of compounds of the corticosterone series is responsible for these changes, while desoxycorticosterone acetate (DCA) is concerned with the regulation and distribution of electrolytes between the extracellular and intracellular fluids.[4]

The normal function of the adrenal glands seems to be under the influence of the anterior lobe of the hypophysis according to the work of Bates *et al.*,[1] Becks *et al.*,[2] and Corey and Britton.[7] The administration of the adrenocorticotropic hormone (ACTH) to hypophysectomized animals induces hypertrophy of the adrenal cortex. Clinical evidence supports this concept. Carnes *et al.*[8] have shown that the output of ACTH by the anterior hypophysis is conditioned by the amounts of adrenocortical hormones which the intact cortices release. Atrophy of the adrenal cortices results from the administration of the unfractionated cortical extract or corticosterone and related compounds.

It is apparent, therefore, that a number of vital body functions, such as carbohydrate and protein metabolism, the water electrolyte balance and kidney functions, and natural body defenses involving lymphatic tissue and antibody production are affected by adrenal cortical secretions.

Cortisone and ACTH have been used extensively for the treatment of arthritis in man. Very little information is available in veterinary literature on the use of these hormones in the treatment of arthritis in domestic livestock. The object of these experiments was to observe the effects of cortisone and ACTH in the treatment of advanced chronic cases of arthritis due to *Erysipelothrix rhusiopathiae* infection. It was doubted that the infectious agent was present in these animals at the beginning of the experiment since the authors were not able to isolate the organism from the joints of swine more than 226 days postexposure, and these animals had been affected for a considerably longer time.

MATERALS AND METHODS

The arthritis was produced experimentally in 5 animals by intravenous injections of cultures of *Ery. rhusiopathiae* as previously described by Sikes *et al.*[14] Two of the arthritic swine had naturally occurring cases and 1 was used in each experiment. All of the arthritic swine had been affected for more than eight months and had been unable to rise without assistance for two to four months prior to hormonal therapy. They were housed indoors in pens with concrete floors and an adequate ration was provided. Corrected erythrocyte sedimentation rates and body weights were determined prior to treatment and at weekly intervals until the experiment was terminated. All hormones were administered subcutaneously.

Three swine with advanced arthritis received 75 mg. of cortisone* daily for four weeks, 50 mg. daily for one week and 25 mg. daily for one week.

In the second group, ACTH** was administered

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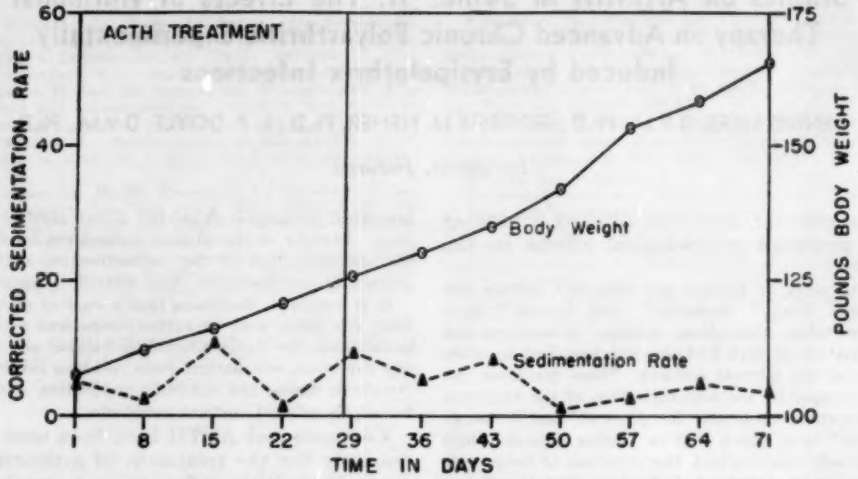
Taken from thesis, "The Pathological Effects of the Bacterium *Erysipelothrix rhusiopathiae* on Swine," submitted to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August, 1954. Present address (Sikes): Department of Pathology and Parasitology, School of Veterinary Medicine, University of Georgia, Athens.

Submitted as journal paper No. 848, Agricultural Experiment Station, Purdue University, Lafayette, Ind.

* Courtesy of Sharpe and Dohme Co.

** Courtesy of Armour and Co.

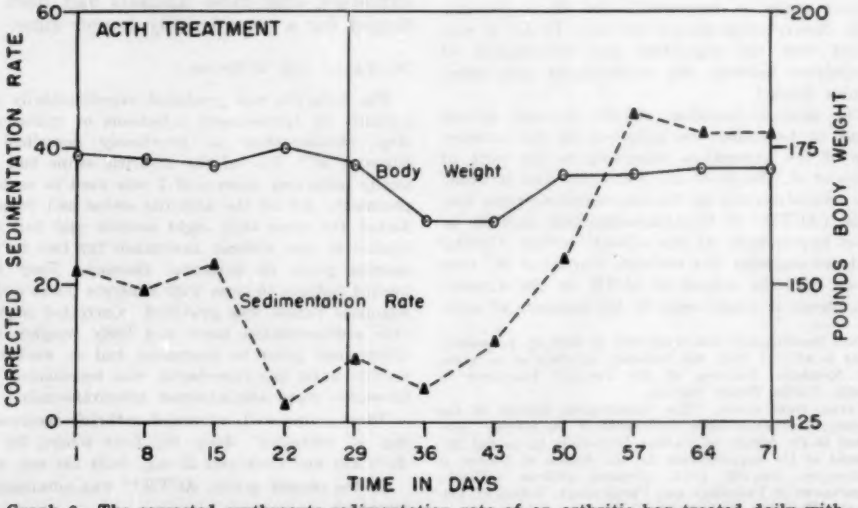


Graph 1—The corrected erythrocyte sedimentation rate of a normal hog treated with 1 unit (U.S.P.) of adrenocorticotropic hormone (ACTH) daily per pound of body weight for one week, ½ unit the second week, and ¼ unit the third and fourth weeks.

to 1 normal hog and 4 which had advanced arthritis. These animals received 1 unit (U.S.P.) of ACTH, in 16½ per cent gelatin, per pound of body weight daily for one week, 1/2 unit daily the second week, and 1/4 unit daily the third and fourth weeks.

RESULTS

Cortisone Therapy in Swine with Chronic Arthritis.—All 3 animals in the first group were able to rise without assistance after the second week of cortisone therapy. A marked clinical improvement in the degree of lameness and stiffness of the joints was observed by the end of the third week of treatment. In all cases, the animals had improved appetites, were more



Graph 2—The corrected erythrocyte sedimentation rate of an arthritic hog treated daily with adrenocorticotropic hormone (ACTH). Note the marked rise in the sedimentation rate soon after withdrawal of treatment.

active, and gained weight during the forty-two days of therapy.

The corrected erythrocyte sedimentation rate of 1 animal was 32 mm. per hour on the first day of therapy, rose to 45 on the seventh day, fell to 24 on the fourteenth day, and to 4 on the twenty-first day. It remained less than 10 for the next two weeks. The authors consider a rate of 10 or less to be within the range for normal swine. The sedimentation rates of the other 2 animals at times were within the range for normal swine but showed marked variation and did not remain within the normal range even though clinical improvement had occurred.

ACTH Therapy in Swine with Chronic Arthritis.—One arthritic animal was slaughtered fifty days after the termination of 28 days of ACTH therapy to examine the adrenal and pituitary glands, while the other 3 arthritic swine were untreated for six weeks and subsequently were given 75 mg. of cortisone daily for four weeks. The normal animal which received ACTH remained unaffected and made excellent weight gains. The corrected erythrocyte sedimentation rate (graph 1) fluctuated between 1 and 9 mm. per hour which is considered to be within the range for normal swine.

The arthritic animals treated with ACTH showed very little if any clinical improvement. In 2 of the animals, the erythrocyte sedimentation rate decreased from 22 mm. per hour to 3 and from 40 to 6 at the end of three and two weeks of therapy,

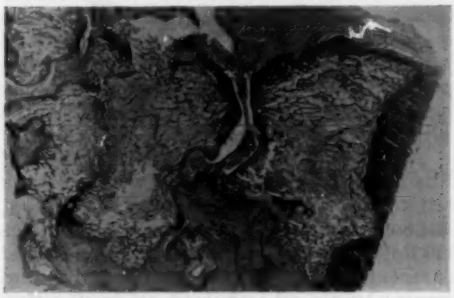
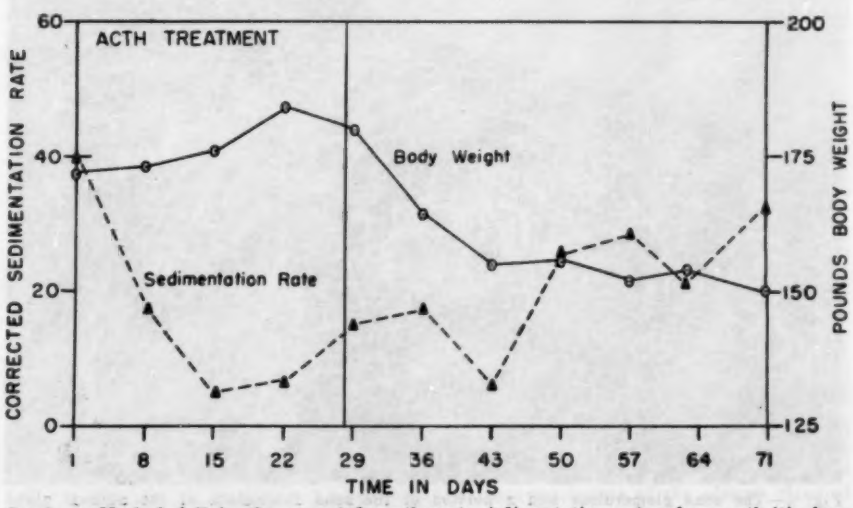


Fig. 1—Thickening of the joint capsule, pannus formation, and destruction of the articulating surfaces in the carpal joint of an arthritic hog. Bacteriological examination of this joint was negative, although the animal became arthritic nine months previously, following exposure to Erysipelothrix rhusiopathiae. x 2.5.

respectively, and remained within the range for normal swine during the fourth week (graphs 2 and 3). Following the discontinuance of therapy, the sedimentation rates rose and in 1 animal was considerably higher than prior to hormonal therapy. The body weight of 1 animal increased slightly while the other remained constant during ACTH treatment; how-



Graph 3—Marked fall in the corrected erythrocyte sedimentation rate of an arthritic hog treated with adrenocorticotropic hormone (ACTH).

ever, a marked decrease in body weight occurred in both animals when therapy was withheld. The changes in the erythrocyte sedimentation rate and body weight of the other 2 arthritic animals followed the same general pattern as described. It was apparent that when ACTH was administered to arthritic hogs, the sedimentation rates were lowered, but at the dosage and for the period of treatment in this experiment, the clinical effect was questionable.

A notable clinical feature with each of the arthritic swine was that within a week after the withdrawal of ACTH therapy, extreme lameness and soreness of the legs and feet returned. The animals would stand for only five to ten minutes and squeal loudly when helped up as if suffering great pain. The concurrent reduction in body weight reflects, in part, a reduced food intake. The normal animal remained unaffected and made excellent weight gains.

Six weeks after witholding ACTH therapy, 3 of the arthritic animals were given 75 mg. of cortisone daily for four weeks. After two weeks their appetites improved, and they gained weight but their arthritic condition had not improved and they were unable to rise without assistance. One animal was slaughtered at this time and the carpal and tarsal joints were found to be enlarged and ankylosed. Radiographs showed osteophytic formations. The flexor tendons appeared to be contracted and little or no fluid was present in the sheaths. Marked fibrosis and thickening of the joint capsules had occurred. A pronounced synovitis, proliferation of the synovial villi and pannus formation were present (fig. 1) along with fibrous ankylosis and erosions of the articular cartilages. No joint fluid was present. Bacteriological examination of these joints gave negative results.

The adrenal glands were slightly enlarged and weighed 10.025 Gm. Microscopic examination revealed that a marked hypertrophy of the zona glomerulosa had occurred (fig. 2 and 3). The kidneys were gray and fibrotic.

The remaining 3 arthritic animals continued to gain weight during the last two weeks of cortisone therapy. They showed evidences of some clinical improvement, were less lame, and moved more freely. However, complete recovery did not occur and the animals could not rise without assistance.

DISCUSSION

Degenerative changes in the adrenal cortex and evidence of rearrangement of the

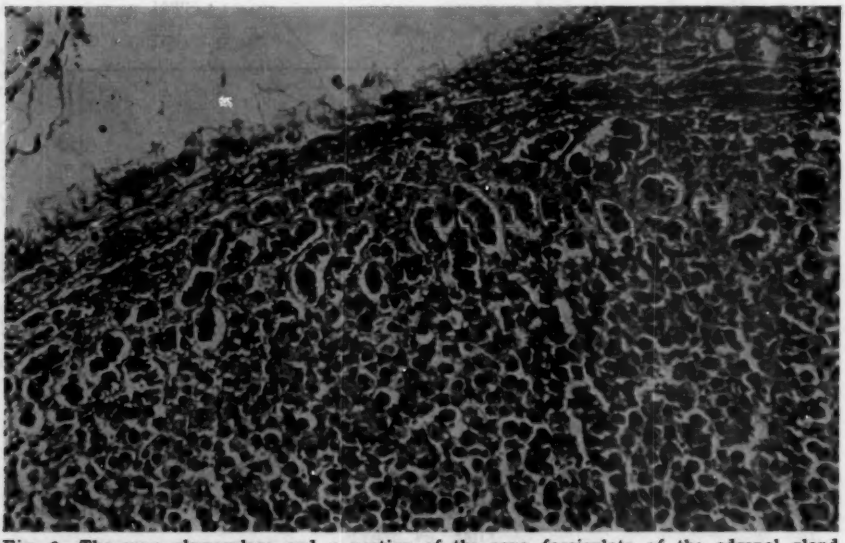


Fig. 2—The zona glomerulosa and a portion of the zona fasciculata of the adrenal gland from an adult hog. x 200.

normal architecture of the adrenal glands and fibrosis of the kidneys were the two most constant pathological changes observed in the viscera of advanced cases of chronic polyarthritis. The importance of these changes in relation to development of the arthritic syndrome is not known. It is known that reduced renal activity delays the excretion of a number of body wastes. Just what effect these by-products of metabolism have on arthritis directly, and on the adrenal gland indirectly, is subject to speculation. It is highly probable that in animals with advanced chronic polyarthritis which have severe damage of adrenal gland and kidney, an interference with normal cortical hormone production and utilization by the animal has occurred. This may account for the continued active development of the arthritic condition after the infectious agent can no longer be isolated from the joints.

Cortisone therapy for six weeks produced a marked clinical improvement in the condition of 3 arthritic swine. There was a remission of soreness and stiffness, and the animals were able to rise without assistance after the third week. They were more active, had improved appetites, and gained weight. Complete recovery was not attained, however. In these animals, erythrocyte sedimentation rates fluctuated within and above the range for normal swine, indicating that sedimentation rate alone may not be reliable for judging clinical improvement. It is important to keep in mind that the course of chronic arthritis is likely to be characterized by periods of exacerbation and remission.

In these 3 animals, adrenal gland and kidney damage may have offset part of the therapeutic value of cortisone; consequently, prolonged therapy with all of the adrenal cortical hormones may sometimes be indicated. Also, in some cases, bone changes may be extensive and clinical improvement may require many months of therapy.

Doyle *et al.*[8] reported marked clinical improvement within five days in 3 shoats which had naturally occurring arthritis and were treated with cortisone (100 mg. daily). When hormonal therapy was discontinued, symptoms recurred within two

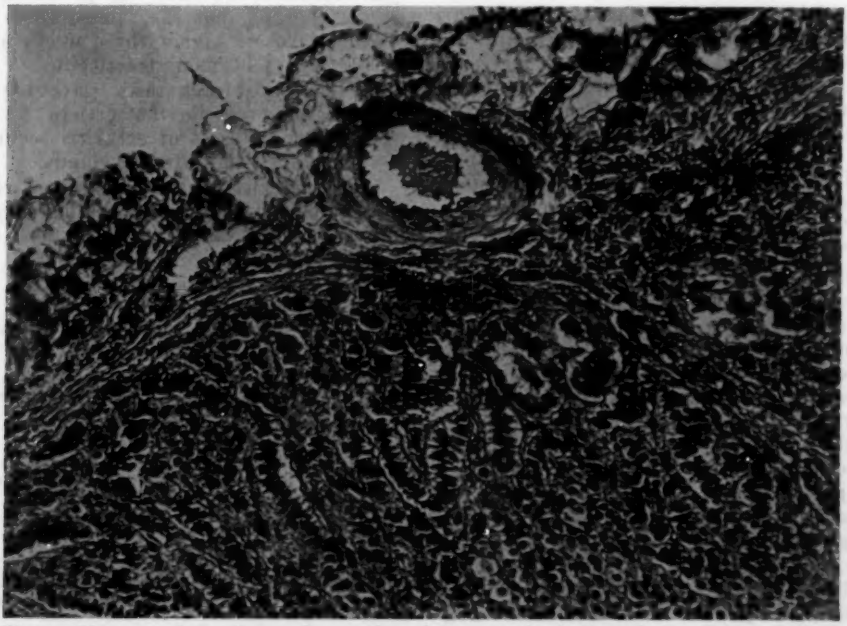


Fig. 3—The zona glomerulosa and a portion of the zona fasciculata of the adrenal gland of an arthritic hog treated with adrenocorticotropic hormone. The zona glomerulosa is apparently markedly hypertrophied. x 200.

to thirty days. The same authors treated 2 arthritic swine with ACTH (40 mg. daily). One animal responded after five days and symptoms returned twenty days post-treatment. The other animal failed to respond.

In the experiments of Doyle *et al.*, the animals responded sooner. However, the dose of cortisone was larger than that administered to the arthritic hogs in this experiment.

Doyle *et al.* reported a remission of symptoms in 1 shoat treated with ACTH followed by a recurrence twenty days later. In the present experiment in which ACTH was used to treat 4 arthritic animals, little or no clinical improvement was observed. However, a reduction in sedimentation rate did occur. Three weeks after the withdrawal of therapy, the sedimentation rates in 3 of the animals rose to a higher level and the animals were considerably worse than before treatment. The appetite was poor and a loss of body weight occurred. In 1 animal which was destroyed to observe the pathological changes, enlargement of the adrenal glands had occurred. The zona glomerulosa appeared hypertrophied (fig. 2 and 3). This suggests hyperactivity of the gland but does not necessarily indicate an increased cortical output. Had there been an increased cortical output, theoretically an improvement in the physical condition of the animals should have taken place, providing severe renal damage had not offset these effects.

From these results, it would seem that ACTH is contraindicated at the dosage used. From the point of pathological considerations, ACTH may be contraindicated in animals with damage to the adrenal gland and kidney, since it stimulates the adrenal glands which in turn influence the adrenal-kidney axis. In animals so affected it would seem that rest and not stimulation of the adrenal glands and kidneys may be beneficial for recovery. This concept is supported in that 3 animals which had lost weight during ACTH therapy, gained weight, had improved appetites, and were less lame after four weeks of subsequent cortisone therapy. The normal animal was unaffected by the ACTH and made excellent weight gains during the period of therapy and thereafter.

Thus it seems that cortisone reduces the severity of the symptoms probably by reducing the soreness, stiffness, and inflammation of the joint structures. It aids in carbohydrate metabolism and indirectly improves the appetite, in spite of adrenal and kidney damage. Since ACTH stimulates the adrenal glands to greater functional activity, theoretically it should improve the condition of the animal unless severe degenerative changes have occurred in the adrenal glands and kidneys prior to hormonal therapy.

SUMMARY

1) The administration of 75 mg. of cortisone subcutaneously daily for four weeks, 50 mg. daily for one week, and 25 mg. daily for one week, produced marked clinical improvement in three advanced chronic cases of arthritis due to *Erysipelothrix rhusiopathiae* infections.

2) At the dosage of adrenocorticotropic hormone (ACTH) used, no appreciable improvement in condition was observed in 4 arthritic swine treated.

3) A marked reduction in the erythrocyte sedimentation rate was produced by ACTH therapy, but the rates rose to the original level or higher three weeks after withdrawal of hormone treatment.

4) The most constant pathological changes observed in the viscera of advanced chronic cases of arthritis occurred in the kidneys and adrenal glands.

5) In arthritic swine with severe renal and adrenal gland damage, ACTH may be contraindicated, since adrenal rest, not stimulation, is needed.

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Vesicular Stomatitis and Vesicular Exanthema Differentiation by Complement Fixation

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IT IS GENERALLY admitted that the differential diagnosis between vesicular stomatitis (VS) and vesicular exanthema (VE) of swine on clinical grounds alone is impossible. There are a number of tests, which are expensive and tedious, that differentiate between these viral agents; however, the complement-fixation test as a diagnostic aid and research tool is of great value for both speed and economy. Unfortunately, the tests used at the present time for VS and VE differ and, therefore, require two sets of techniques, standards, and reagents to establish a serological diagnosis. A test in which an identification and differentiation can be made in one operation is highly desirable. These considerations and the apparent increase of VS occurring in swine in various parts of the United States stimulated the present study. Because VS is not an enzootic disease of swine in California, this work has been done in our laboratory with inactivated virus and the observations are based on materials which are well controlled. The well-defined results obtained with VS, which are perhaps better than those usually encountered under comparable conditions with the virus of VE, suggest that the method could be of great value with material from field outbreaks.

METHODS

The VS virus and the homologous antiserums were obtained through the courtesy of Dr. L. O. Mott of the Animal Disease and Parasite Research Branch, ARS, U.S.D.A. Both immunologically distinct types of VS virus (type Indiana, strain Fort Lupton, and type New Jersey, strain Concan) were cultivated and represented a 10 per cent suspension of chorioallantoic membranes of the infected chicken embryonating eggs. The suspensions were inactivated with ultraviolet light.[1] The two strains of virus were shipped in dry ice by air and reached the laboratory the following day. Upon receipt, the viruses were stored at −15 F. and thawed just before use. The VS antiserums were obtained from hyperimmunized swine, and both types of antiserums were tested by the complement-fixation test by the ARS laboratory and were found to contain sufficient complement-fixing antibodies.

Vesicular exanthema virus and the respective type-specific, swine hyperimmune serums were prepared and proper dilutions employed in the procedure as previously reported.[2]

Briefly, the test consisted of doubling dilutions of the antigen (1 through 160) in the presence of various agents. Controls for all reagents were included with each test. Since the high concentration of third component (C_3) of swine serums enhanced the complement titer of guinea pig serums, each swine serum employed in the test was included in the guinea pig complement titration, and the dose of complement used was carefully adjusted to contain 1 full unit. The amounts of the various reagents used were: (1) virus, 0.25 ml. of the various dilutions; (2) swine antiserum, 0.25 ml. of a dilution which has been previously determined for optimum antibody concentration; and (3) complement, 0.25 ml. or less in a dilution as previously determined to contain 1 full unit in presence of 0.25 ml. of each swine serum in the test. Where the volume was less than 0.75 ml. or a reagent was omitted, the total volume was made up with 0.9 per cent NaCl.

Fixation of complement was allowed to take place in an ice chest at 4 C. for eighteen to twenty-four hours. One-half milliliter of the hemolytic system, which consisted of equal parts of amboceptor containing 4 units per 0.25 ml. and a 4 per cent sheep red blood cell suspension, was added to each tube and incubation was continued for thirty minutes at 37 C. in a water bath. Readings were made immediately thereafter.

RESULTS

When both types of VS virus, employed as crude 10 per cent suspensions of chorioallantoic membranes, were tested against 10 or 20 per cent homologous swine antiserums, clear-cut fixation of complement to a high degree resulted, but a differentiation between the New Jersey and Indiana types was not evident. Also of interest is the marked cross fixation between both types of VS virus and types A_{48} and B_{51} VE, diluted antiserums (table 1). Repetition of the test with the same materials, but clarifying the antigens with Lloyd's reagent before testing, markedly decreased

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the cross fixation between VS antigens and VE swine antiserums, which suggests that much of the fixation was probably nonspecific. Similar nonspecific reactions were observed in experiments dealing with crude antigens and with chicken and swine embryonic tissues following clarification.[3]

Type-specific fixation has been obtained with type B, VE virus as an antigen using all four VE and both types of VS swine antiserums, even though the latter were employed in high concentrations.

Since a differentiation between the two types of VS virus was not possible when high concentrations of antiserums and virus were used in the presence of 1 unit of complement, a number of experiments were carried out on the factors influencing the degree of fixation between VS virus and their antiserums. It was found that differentiation between the two types could be demonstrated (1) by keeping the antigen constant (10 % suspension) and varying, by dilution, the amount of serums or antibody in the reaction and (2) by keeping the serums at a predetermined concentration (1 %) and varying the amount of antigen in the reaction. Of these two procedures, the latter would be far more desirable to adapt to this test because the test uses as antigen unknown material which is submitted to the laboratory for identification. It was also evident that a far greater degree of fixation was obtained by this method using VS antigens and antiserums than is usually encountered in VE under optimum conditions. With these points in mind, a series of experiments were conducted using a predetermined and standardized level of swine antiserums with doubling dilutions of a 10 per cent antigen, which would be comparable to the prepared sample as it is obtained from the field, and conducting the tests in the presence of 1 and 2 full units of complement. As indicated in table 2, type-specific fixation of complement could be clearly demonstrated and differentiation between the types was far more effective when the complement was increased from 1 to 2 full units.

Since fixation of complement, even in the presence of large quantities of VE antigen and the homologous antiserums, is "washed out" with 2 full units of complement and no significant cross fixation between the four types of VE antigens (A_{48}, B_{51}, C_{52}, and D_{53}) was found in the limited amount of experimentation with VS antiserums, it appeared that a single test to include VS can be incorporated in the routine testing program. Therefore, to detect and differentiate VE or VS by this tech-

TABLE 1 — Fixation of Complement in Tests Using Crude 10 Per Cent Suspensions of Types New Jersey and Indiana Vesicular Stomatitis Virus[1] and Vesicular Stomatitis and Vesicular Exanthema Swine Antiserums in the Presence of 1 Unit of Complement[2]

ANTIGEN[3]	ANTISERUMS			ANTIGEN DILUTION				
	DIS.	TYPE	%	10	20	40	80	160
10% New Jersey V. S. (concan)		NORM.	4	—	—	—	—	—
	V. E.	A_{48}	2	3+	3+	4+	4+	4+
		B_{51}	4	3+	3+	4+	+	+
		C_{52}	2	—	—	—	—	—
		D_{53}	20	—	—	—	—	—
	V. S.	N.J.	20	4+	4+	4+	3+	3+
		IND.	10	4+	4+	4+	4+	4+
10% Indiana V. S. (Ft Lupton)		NORM.	4	—	—	—	—	—
	V. E.	A_{48}	2	4+	4+	4+	4+	4+
		B_{51}	4	2+	2+	—	—	—
		C_{52}	2	—	—	—	—	—
		D_{53}	20	—	—	—	—	—
	V. S.	N.J.	20	4+	4+	4+	+	+
		IND.	10	4+	4+	4+	4+	4+

[1] Ten per cent suspension of infected chorioallantoic membranes of chicken embryonating eggs. [2] Using a 10 per cent suspension of vesicles infected with type B_{51} vesicular exanthema virus as antigen within the same test showed a specific fixation with type B antiserum without cross fixation with other vesicular exanthema or vesicular stomatitis antiserums. [3] Antigens centrifuged at 3,000 r.p.m. for ten minutes before use.

TABLE 2 — Specificity of Types in Complement Fixation Using 1 and 2 Full Units of Complement

ANTIGEN	ANTISERUMS		Fix with 1 full unit of complement				
	TYPE	%	10	20	40	80	160
10% N.J. V.S. (Concan)	N.J.	8	4+	4+	4+	4+	4+
	Ind.	8	4+	4+	4+	3+	—
10% Ind. V.S. (Ft. Lupton)	N.J.	8	4+	3+	—	—	—
	Ind.	8	4+	4+	4+	4+	4+
			Fix with 2 full units of complement				
10% N.J. V.S. (Concan)	N.J.	8	4+	4+	4+	4+	4+
	Ind.	8	4+	4+	—	—	—
10% Ind. V.S. (Ft. Lupton)	N.J.	8	—	—	—	—	—
	Ind.	8	4+	4+	4+	4+	3+

nique, an unknown antigen is titrated with normal and all four immunologically distinct types of VE antiserums in the presence of 1 full unit of complement and with the two types of VS antiserums in the presence of 2 full units of complement.

In another modification in which fixation was carried out at 37 C. for thirty minutes in a water bath in place of fixation overnight, good fixation and differentiation was obtained with the Indiana virus but could not be obtained with the New Jersey virus. This may have been due to the quality of the reagents, however, the fixations were found to be far superior with the prolonged incubation period.

DISCUSSION

It must be emphasized that the results recorded were obtained with material from an egg-propagated VS virus. Furthermore, only one strain of each type of the virus was employed. Confirmation, provided by the use of both infected material from hogs suffering from the disease and diversified strains, is greatly desired. This point can not be overemphasized, judging from the experience obtained with an apparent variation in strain characteristics of VE which is routinely collected and submitted to this laboratory for testing, as well as the experience of the English workers with a number of strains of the virus of foot-and-mouth disease.[4,5] In view of the clear-cut results recorded here, this test could be used as an additional diagnostic tool with justifiable optimism.

Points worthy of note are the marked differences observed in the degree of fixation between VE and VS viruses and the similarity of the serological behavior of VE to foot-and-mouth disease virus. Like foot-and-mouth disease, VE shows a considerable cross reaction between the immunological types. Brooksby [6] regards overlapping with foot-and-mouth disease as manifestations of a fundamental property of the virus due to the presence of a common antigen or as a degree of similarity between the type antigens which allows for this cross fixation at high antibody titers. Brooksby also noted a two- or threefold greater fixation with VS virus than with foot-and-mouth disease virus and emphasized the parallel correlation of the complement-fixing antibodies of these two diseases to the behavior of the neutralizing antibodies. Other evidence of similarity between the antigenic components with regard to cross neutralization and anamnestic response by heterologous types of foot-and-mouth disease virus has been observed with the VE virus.[3,4] From the data and experience obtained, the behavior of VE virus in serological reactions seems to be more closely allied to the behavior of foot-and-mouth disease virus than it is to VS.

SUMMARY

A method of detecting and differentiating vesicular exanthema (VE) and vesicular stomatitis (VS) is described. The complement-fixation reaction is type specific for the two strains of VS virus which was propagated in embryonating chicken eggs. No serious cross fixation was evident with types A_{48}, B_{51}, C_{52}, and D_{53} VE virus.

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The Histochemical Nature of *Anaplasma Marginale*

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Anaplasma marginale Theiler, 1910, is generally described as a small, rounded body consisting of a dense mass of chromatin, devoid of cytoplasm, and multiplying by fission.[1,2] The bodies have been considered by various investigators to represent a phase in the life cycle of the Piroplasmidae, a form of Howell-Jolly body, a reaction product to a virus, a degeneration product of the erythrocyte, and a protozoan parasite. The literature on this subject has been reviewed thoroughly by Du Toit [1] and Dikmans.[3] There have also been many papers on the morphology of the Anaplasma bodies, including a recent electron microscope study in which elementary bodies were described,[4] but there has been only one report dealing with their histochemistry. Penha [5] used the Feulgen technique to demonstrate desoxyribonucleic acid in the Anaplasma bodies and showed that they could be differentiated from Howell-Jolly bodies on that basis.

This paper reports the results of detailed histochemical studies of Anaplasma bodies.

Materials and Methods

Blood smears were prepared from 2 cows experimentally infected with blood containing *A. marginale*. These smears showed approximately 50 per cent of the erythrocytes to be carrying marginal bodies (fig. 1). Diagnosis of anaplasmosis was confirmed at necropsy.

Unfixed and fixed blood smears were employed in each histochemical test. The following fixatives were used: Zenker's fluid, acetone, 10 per cent formalin, methyl alcohol, and Carnoy's fixative. The smears were fixed for one hour at 4 C. and, prior to staining, were washed in tap water for ten minutes.

The substances investigated and the histochemical tests utilized are listed in table 1. Neutrophils in the smears provided positive-staining controls for many of the tests, and kidney cortex was used as the control in the alkaline phosphatase test. The following negative-staining controls were employed: unhydrolyzed smears with the Feulgen reaction, smears digested with ribonuclease in the test for ribonucleic acid, and substrate lacking phosphate in the alkaline phosphatase test.

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Results

The Anaplasma bodies showed a positive pink color in the Feulgen test for desoxyribonucleic acid (fig. 2). Unhydrolyzed control smears were negative (fig. 3). Leukocyte nuclei reacted like the Anaplasma bodies (fig. 2 and 3). The Anaplasma bodies also exhibited positive staining in the methyl green-pyronine test for ribonucleic acid (fig. 4). The bodies stained red and this color could be prevented by incubating smears in a solution of ribo-

TABLE 1 — Substances Investigated and Histochemical Methods Employed in the Study of Anaplasma Marginale

Substances	Methods
Desoxyribonucleic acid	Feulgen [6]
Ribonucleic acid	Methyl green pyronine and ribonuclease [7]
Protein	Ferrocyanide [8]
Arginine	Sakaguchi [9]
Tyrosine	Millon [10]
Polysaccharide complexes	Periodic acid-Schiff [11]
Organic iron, inorganic iron	Prussian blue [12]
Calcium	von Kossa [13]
Lipids	Sudan black B [13]
Fat peroxides	Stable or M. Nadi [6]
Dehydrogenase	Tetrazolium [14]
Peroxidase	Benzidine [8]

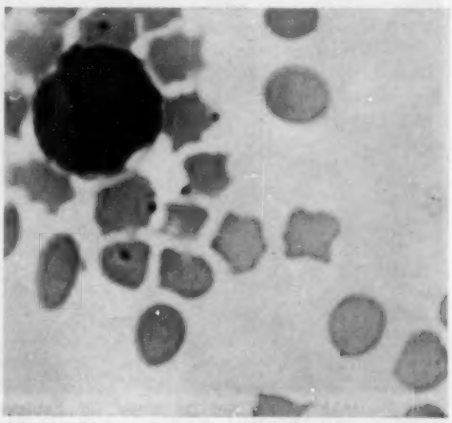

Fig. 1—Giesma stain of unfixed blood smear showing Anaplasma bodies in the erythrocytes. x 1800.

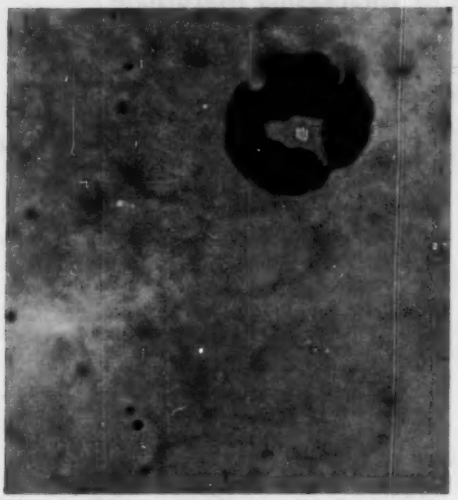

Fig. 2—Feulgen reaction for desoxyribonucleic acid. Anaplasma organisms and neutrophil nucleus show positive reactions. Methyl alcohol fixation, x 1800.

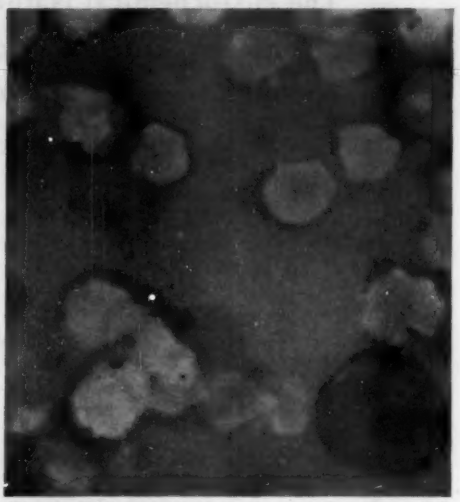

Fig. 4—Methyl green-pyronine test for ribonucleic acid. Anaplasma bodies and leukocyte cytoplasm showed positive red staining. Carnoy's fixative, x 1800.

nuclease before staining (fig. 5). Leukocyte cytoplasm reacted like Anaplasma bodies in this test and leukocyte nuclei stained bluish green and were unaffected by enzyme digestion. Complete loss of stainability in both Anaplasma bodies and leukocytes was obtained after extraction in 5 per cent trichloroacetic acid.

The Anaplasma bodies demonstrated positive blue staining in the ferrocyanide

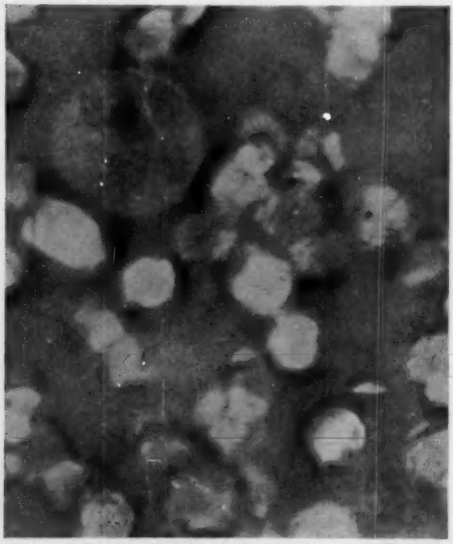

Fig. 3—Unhydrolyzed control smear for Feulgen reaction. Note negative reactions of Anaplasma bodies and neutrophil nucleus. Methyl alcohol fixation, x 1800.

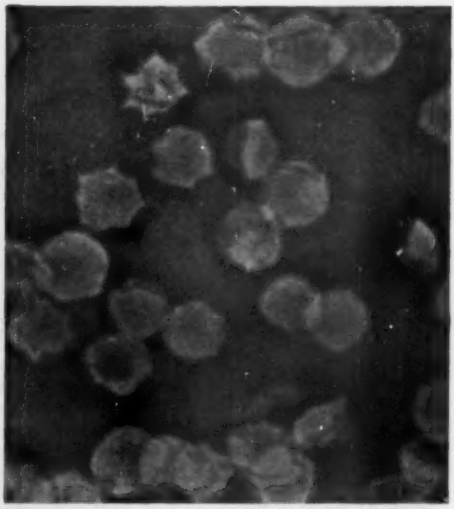

Fig. 5—Ribonuclease-digested control smear for methyl green-pyronine test for ribonucleic acid. Note absence of staining of Anaplasma bodies. Carnoy's fixative, x 1800.

test for protein, but tests for arginine and tyrosine were negative. No polysaccharide complexes could be demonstrated in the bodies by the periodic acid-Schiff method. Schiff-positive control staining was observed in the myeloid granules in the same preparations (fig. 6).

The Prussian blue test for organic iron reacted with minute, blue coloration of Anaplasma bodies and leukocyte nuclei. The tests for inorganic iron and calcium were negative. Lipids (fig. 7), fat peroxides, and peroxidase could not be demonstrated in the Anaplasma bodies and positive control staining of these substances was observed in the myeloid granules (fig. 7). The bodies were also negative for dehydrogenase and alkaline phosphatase. Control sections of bovine kidney cortex showed positive staining for alkaline phosphatase and tissues incubated in substrate lacking phosphate reacted negatively.

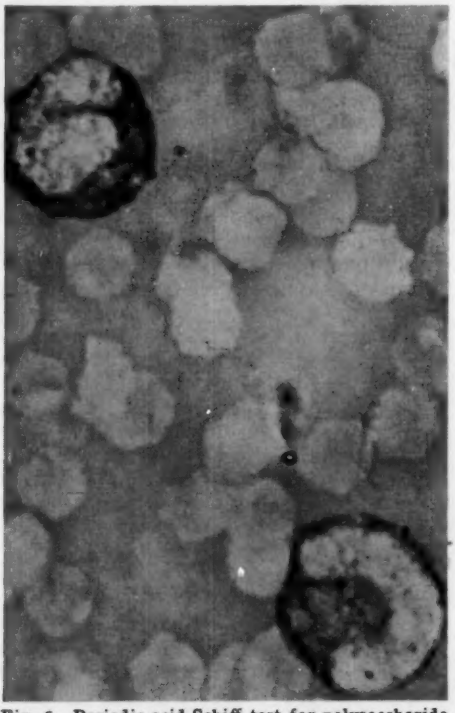

Fig. 6—Periodic acid-Schiff test for polysaccharide complexes. This shows negative staining of Anaplasma bodies and positive control staining of myeloid granules. Zenker's fixative, x 1800.

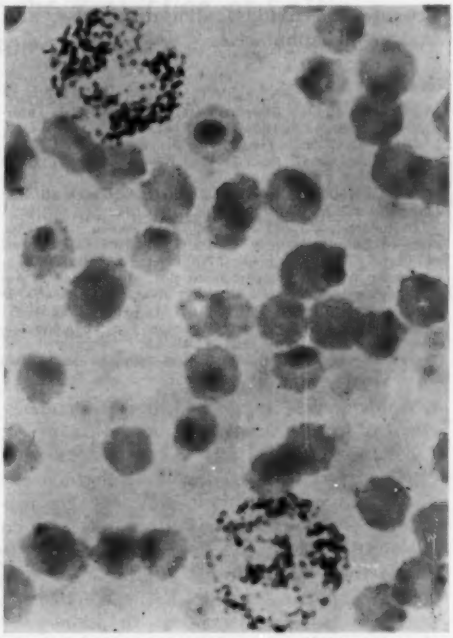

Fig. 7—Sudan black B stain for lipids. Note absence of staining in Anaplasma bodies and positive control staining of myeloid granules. Unfixed smear, x 1800.

DISCUSSION

The positive histochemical reactions for desoxyribonucleic acid, ribonucleic acid, organic iron, and protein in *A. marginale* seem to indicate that these bodies are animate particles containing nuclear chromatin. Their particular chemical composition is not consistent with their being degradation products of erythrocytes, Howell-Jolly bodies, or staining artefacts. However, on the basis of the histochemistry of the Anaplasma bodies alone, no differentiation can be made as to whether or not they represent bacteria, viruses, or Protozoa.

SUMMARY

Anaplasma marginale in blood smears were studied by histochemical techniques. The Anaplasma bodies showed positive reactions for desoxyribonucleic acid, ribonucleic acid, protein, and organic iron. Negative-staining results were obtained for arginine, tyrosine, polysaccharide complexes, inorganic iron, calcium, lipids, fat

peroxides, peroxidase, dehydrogenase, and alkaline phosphatase.

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An Immunogenic Agent for the Protection of Cattle Against *Leptospira Pomona*

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BOVINE leptospirosis is a disease which presents an important control problem because of the large numbers of viable organisms shed in the urine of infected or convalescent animals. The resulting high level of infectivity more than compensates for the relatively low incidence of clinical disease. Bovine leptospirosis is manifested by a variety of symptoms including abortion, pyrexia, hemoglobinuria, and sudden death. A large number of cases, however, are of the subclinical or inapparent type. Animals with either apparent or inapparent infection may develop into transitory urinary shedders in the clinically convalescent or recovered phase. Those with inapparent infection may shed organisms which cause apparent clinical symptoms in the next host. It is important, then, for satisfactory control of the disease, that the elimination or prevention of clinical symptoms be accompanied by the prevention of the urinary carrier state.[1-3]

The experience of research workers and clinicians in the search for a specific therapeutic agent for the treatment of bovine leptospirosis has been varied and disappointing. Hall *et al.*[4] undertook to evaluate a series of antibiotics in an outbreak of leptospirosis in man. Their work indicated that the antibiotics used were of little value. Similar indications have been reported by veterinarians, although some workers have attributed recoveries to the use of various antibiotics. Hoag and Bell,[5] in *in vitro* studies, found evidence of a serotype specificity to the action of streptomycin. This may be an explanation for the varied results encountered in the antibiotic treatment of bovine leptospirosis.

The diagnosis of leptospirosis is difficult and time-consuming. Serological methods depend upon the demonstration of antibody titers in paired serum samples.[6-9] Increasing titers in serial serum samples taken at a minimum ten-day interval indicate recent leptospiral infection. Bacteriological techniques are laborious and difficult because of the fastidious growth requirements of the organism. It can be readily understood, therefore, that by the time a diagnosis is made by serological or bacteriological or by both methods the patient is either recovered, convalescent, or dead.

Active immunization of susceptible cattle in an enzootic area or in an infected herd appears to be the logical approach to the control or elimination of bovine leptospirosis.[10-11] The immunizing agent should be one that will prevent the development of urinary carriers. The use of attenuated organisms might prevent development of clinical symptoms but not prevent the development of urinary carriers. Furthermore, virulence of the organisms may be increased in such carriers with ensuing possibilities of future explosive outbreaks in susceptible hosts. The immunizing agent should be inexpensive so that owners can have their herds routinely immunized in order to prevent the hidden economic losses which may be spread over a period of months or years.

With these thoughts in mind, studies on a leptospiral immunogenic agent were begun. The results of these studies form the basis of this report.

MATERIALS AND METHODS

1) *Preparation of the Immunogenic Agent.*—*Leptospira pomona* S91* was grown on Stuart's liquid medium [12] in Ehrlenmeyer flasks at 30 C. for ten to fourteen days. After incubation, the contents of the flasks were pooled and the total volume measured. One-tenth normal acetic acid was added in an amount equal to 3 per cent of the volume of the pooled material. The acidified culture was heated to 100 C. until a heavy, whitish curd of protein material was formed (10 to 15 minutes). After cooling to room temperature with running tap water, the precipitate was removed by filtration or centrifugation and resuspended in a small amount of distilled water. One-tenth nor-

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* Obtained from the Veterinary Division, Army Medical Service Graduate School, Washington, D. C.

mal NaOH was then added to the resuspended precipitate until complete solution was obtained. The reconstituted material was then brought to one-fifth of the original pooled volume by the addition of distilled water and preserved with merthiolate† at a final concentration of 1:10,000. Two parts of this final concentration were added to 3 parts of an adjuvant composed of lanolin and liquid petrolatum (2 parts petrolatum, light grade, N.F. and 1 part lanolin).[13] This mixture, designated as the immunogenic agent, was shaken well and stored at approximately 5 C. until use. At the time of use, the immunogenic agent was warmed until liquefied and shaken well before injection.

2) *Use of the Immunogenic Agent in Cattle.*—Experiment 1.—Dairy calves, 10 to 12 weeks old and negative to the microscopic agglutination-lysis test[14] to *Leptospira pomona, Leptospira grippotyphosa, Leptospira sejroe,* and *Leptospira canicola*[15, 16] were selected for uniformity in size. Eight calves were injected subcutaneously in the cervical region with 5 ml. of the immunogenic agent two weeks after its preparation. Seven days later, 4 of these calves were similarly reinjected. Five uninjected calves were used as controls. Two months after the last injection date, all 13 calves were challenged.

Leptospira pomona 285‡ was used as the challenge material. The virulence of the strain was increased for calves by inoculating a sample of blood from a moribund hamster (inoculated 3 days previously with a culture of the organisms) into a 7-week-old calf. The hamster blood was diluted in saline to 10^{-1} and inoculated intramuscularly in the thigh region of the calf. At the first temperature rise in the calf, 30 ml. of venous blood was drawn and similarly inoculated into a second calf. Passage in this manner was made through 6 calves until clinical symptoms were consistently produced as early as the fourth day. Virulence in the last 2 of the serial passage calves was such that death occurred on the ninth and fifteenth days, respectively. *Leptospira pomona* were cultured from all blood used in these passages. Blood from the sixth passage calf, diluted with normal saline to 10^{-1}, was used for the challenge material. Each calf in the experiment received 10 ml. of this challenge material intramuscularly in the thigh region.

Rectal temperatures were taken twice daily for fifteen days after challenge (at 8 a.m. and 4 p.m.). General examination of each calf was made at this time. Blood samples for the agglutination-lysis test were drawn at the time of vaccination, challenge, and on the seventh, twelfth, twenty-third, thirty-third, fortieth, and fifty-third day after challenge. Blood samples were also taken aseptically each day for fifteen days after challenge for culturing. These samples were immediately inoculated into both Fletcher's and Schüffner's mediums.[28] The cultures were incubated at 30 C. for thirty days before being discarded as negative.

Samples of urine were drawn weekly starting on the twelfth day after challenge. One-half milliliter of the urine obtained from each calf was inoculated into each of 4 hamsters. Blood was obtained from the hamsters by cardiac puncture on the fourth day and cultured in Fletcher's and Schüffner's mediums.

All surviving animals were destroyed fifty-three days after challenge. Complete portmortem examinations were made, and portions of the kidneys were taken for culture. The kidney tissue was ground with a TenBroeck-type tissue grinder and inoculated into hamsters, as well as directly into Fletcher's and Schüffner's mediums.

Experiment 2.—A second lot of the immunogenic agent was prepared for use according to the procedure previously described. However, it was injected into calves one month after preparation rather than two weeks, as in the first experiment.

A group of dairy calves, 6 to 8 weeks of age, were selected for the second experiment. They were of uniform size and were negative for leptospiral agglutinating antibodies. Four calves were given a single dose of the immunogenic agent, 4 received two doses at a seven-day interval, and 2 were left as controls.

All calves were challenged four weeks after the last inoculation date of the multiple dose group. The challenge material was prepared as in experiment 1.

† Thimerosal, Eli Lilly and Co.

‡ Obtained from the Veterinary Division, Army Medical Service Graduate School, Washington, D. C.

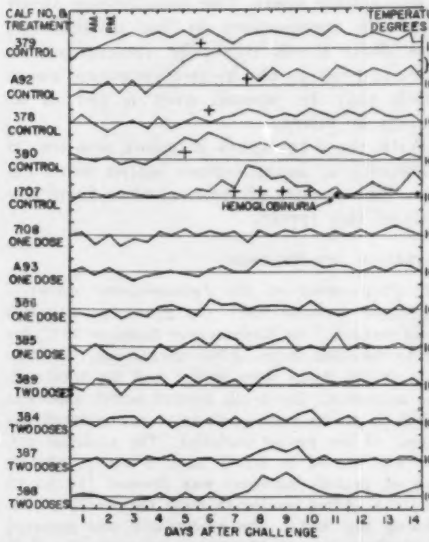

Fig. 1—Temperature charts, following challenge with Leptospira pomona, of calves injected with the immunizing agent and of control calves in experiment 1. Plus signs indicate isolation of L. pomona from the blood.

Blood and urine cultures were made, and serological tests were run as described in experiment 1. Clinical examinations were made throughout the course of the experiment and rectal temperatures were recorded for the first fifteen days after challenge. Postmortem examinations and cultures were made, as in experiment 1, on the fifty-fifth day after challenge.

RESULTS

The results of experiment 1 are summarized in figure 1 and table 1.

Fever and anorexia were produced in all control calves by the challenge material. Leptospiremia, determined by blood culture, developed in all controls and all became urinary shedders. On necropsy of the control calves fifty-three days after challenge, *L. pomona* was isolated from the kidneys which consistently showed a severe interstitial nephritis. This varied from a severe chronic to the peracute form found in calf 1707. This calf had developed hemoglobinuria and had died on the fifteenth day.

Temperatures remained normal after challenge in the calves which had received the immunogenic agent except for a transitory rise in 3. There was no correlation of temperature rise with the number of doses of immunogenic agent received. The calves which had received the immunogenic agent did not develop leptospiremia or leptospiruria and none became urinary shedders. They were entirely normal on postmortem examination and kidney material did not yield Leptospira on culture.

All controls developed a serum titer against *L. pomona* antigens on the agglutination-lysis test. The calves receiving the immunogenic agent did not develop agglutinating antibody either before or after challenge, except calf 385 which developed a titer at the fortieth day after challenge.

The results of experiment 2 are summarized in figure 2 and table 2.

The controls developed clinical signs of leptospirosis with anorexia, fever, and hemoglobinuria. *Leptospira pomona* was isolated from the blood during the febrile stage and both controls became urinary

TABLE 1—Results of Challenging Immunized and Control Calves with Leptospira Pomona—Experiment 1

Calf No. and treatment		D	D+7	D+12	D+23	D+33	D+40	D+53	Remarks (postmortem)
379	Serology	—	100	1,600	400	100	100	100	Kidney lesions
Control	Urine culture	ND	ND	—	+	—	—	ND	Pos. kidney culture
A92	Serology	—	—	400	6,400	25,600	25,600	102,400	Kidney lesions
Control	Urine culture	ND	ND	—	+	+	+	ND	Pos. kidney culture
378	Serology	—	—	6,400	6,400	25,600	102,400	102,400	Kidney lesions
Control	Urine culture	ND	ND	+	+	+	+	ND	Pos. kidney culture
380	Serology	—	—	6,400	6,400	102,400	25,600	25,600	Kidney lesions
Control	Urine culture	ND	ND	—	+	—	—	ND	Pos. kidney culture
1707	Serology	—	—	400		Died on fifteenth day.			Kidney lesions
Control	Urine culture	ND	ND	+		Died on fifteenth day.			Pos. kidney culture
7108	Serology	—	—	—	—	—	—	—	Normal
One dose	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
A93	Serology	—	—	—	—	—	—	—	Normal
One dose	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
386	Serology	—	—	—	—	—	—	—	Normal
One dose	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
385	Serology	—	—	—	—	—	400	1,600	Normal
One dose	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
389	Serology	—	—	—	—	—	—	—	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
384	Serology	—	—	—	—	—	—	—	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture
387	Serology	—	—	—	—	—	—	—	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
388	Serology	—	—	—	—	—	—	—	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	ND	Neg. kidney culture

Serology recorded as highest agglutination-lysis serum titer. — = No agglutination; + = isolation of *L. pomona*; ND = not done; D = day of challenge.

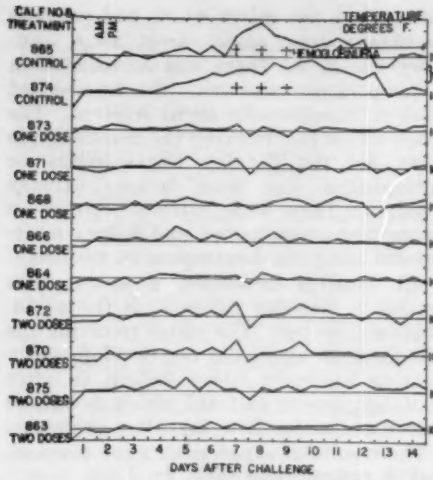

Fig. 2—Temperature charts, following challenge with Leptospira pomona, of calves injected with the immunizing agent and of control calves in experiment 2. Plus signs indicate isolation of L. pomona from the blood.

shedders. Postmortem lesions were consistent with the clinical findings. Isolation of *L. pomona* was made from the kidney material taken at the autopsy of each control.

The calves which had received the immunogenic agent remained normal clinically and were negative on postmortem examination and culture. Leptospiruria and leptospiremia did not develop in any of these calves.

Both controls developed high agglutination-lysis antibody titers following challenge. The calves injected with the immunogenic agent developed titers from 1:100 to 1:400 as a result of the injection but only slight rises in titers resulted from injection of the challenge material.

DISCUSSION

The high antigenicity of soluble antigens has been demonstrated by the work of Ezell *et al.*[17] Since the studies being reported were begun, Larson *et al.*[18] have reported on the use of antigens of the soluble type as immunogenic material in mice. Their work supports the findings reported here in which agglutinating antibodies were not correlated with the presence of immunity. The possible use of soluble antigens for immunogenic purposes in many diseases seems hopeful. The detection of agglutinating antibodies is used in many diagnostic techniques and the differentia-

TABLE 2—Results of Challenging Immunized and Control Calves with Leptospira Pomona—Experiment 2

Calf No. and treatment		D	D+5	D+12	D+23	D+37	D+46	D+55	Remarks (postmortem)
865	Serology	—	400	25,600	6,400	1,600	1,600	102,400	Marked kidney lesions
Control	Urine culture	ND	ND	—	+	—	—	+	Pos. kidney culture
874	Serology	—	—	400	25,600	1,600	6,400	6,400	Slight kidney lesions
Control	Urine culture	ND	ND	—	+	—	+	+	Pos. kidney culture
873	Serology	400	400	1,600	1,600	1,600	1,600	1,600	Normal
One dose	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
871	Serology	100	100	100	100	400	100	400	Normal
One dose	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
868	Serology	400	400	400	400	1,600	1,600	1,600	Normal
One dose	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
866	Serology	400	400	400	400	1,600	1,600	1,600	Normal
One dose	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
864	Serology	100	100	400	400	400	400	400	Normal
One dose	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
872	Serology	400	400	6,400	6,400	1,600	1,600	1,600	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
870	Serology	400	400	1,600	400	400	400	400	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
863	Serology	400	400	400	1,600	1,600	1,600	1,600	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture
875	Serology	400	400	400	400	400	100	400	Normal
Two doses	Urine culture	ND	ND	—	—	—	—	—	Neg. kidney culture

Serology recorded as highest agglutination-lysis serum titer. — = No agglutination; + = isolation of *L. pomona*; ND = not done; D = day of challenge.

tion of artificially immunized, naturally immunized, and infected animals is a difficult task at best. The widespread use of agglutinogenic antigens as immunizing agents often makes this differentiation impossible. The use of an agent which produces a satisfactory immunity, yet produces little or no confusing agglutinating antibody response, simplifies the problem. The results presented here indicate that the immunogenic agent prepared and used in these studies produces a satisfactory immunity without seriously interfering with diagnostic techniques. It could, therefore, be useful in a control program.

The calves in experiment 1 did not develop agglutinating antibodies after receiving the immunogenic agent, whereas the calves in experiment 2 developed low titers (none higher than 1:400). This difference in response is probably attributable to a difference in antigenicity of the two lots of the immunogenic agent. Low titers, such as developed in the calves in the second experiment, would not interfere with routine agglutination-lysis tests used for diagnosis or survey purposes. In both experiments, however, the immunogenic agent produced an immunity which appeared to be effective in protecting against bacteremia, bacteriuria, clinical illness, and the chronic form of the disease. The control calves, on the other hand, presented all of these signs of infection by *L. pomona*.

Another difference in the two experiments lies in the antibody response of the immunized calves after challenge. The calves in the second experiment showed a small increase over their prechallenge low titers, while the immunized calves in the first group, except one, showed no such response. One explanation for this variation may be that the second lot of challenge material had a greater virulence than that used in the first experiment. The difference in the ages of the two groups of calves may have also been a factor.

Summary

A soluble antigen prepared by the acid-heat extraction of living cultures of *Leptospira pomona* was used as an immunogenic agent. The results of its use in calves indicate that it has the following characteristics: (1) it is inexpensive, simple in preparation, and is reproducible; (2) it produces little or no agglutination-lysis antibody (no higher response in calves than 1:400 serum titer in the test system); and it (3) produces a satisfactory immunity to *L. pomona* as indicated by the absence of leptospiremia, leptospiruria, and other pathological or clinical evidence of *L. pomona* infection.

Further studies of the duration of immunity, cross protection against other leptospiral serotypes, laboratory immunogenic titrations, and storage qualities of this product are to be undertaken.

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Oxytetracycline in Bovine Mastitis
I. Treatment of Mastitis

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INFECTIOUS bovine mastitis is one of the most important disease problems of dairy cattle. Oxytetracycline, introduced in 1950, is one of the recent agents which has been used as an aid in mastitis control. The purpose of this work is to evaluate the efficacy of treatment of infectious bovine mastitis with oxytetracycline in controlled clinical trials.

LITERATURE REVIEW

Finlay *et al.*[4] reported in 1950 the isolation of oxytetracycline (terramycin®) from *Streptomyces rimosus*. The first reports on the use of the drug in treatment of infectious bovine mastitis appeared in 1951. Tableman[10] treated 8 animals with oxytetracycline udder infusion ointment. All became clinically normal within seventy-two hours, but he did not identify the etiological agents. Cromley and Hagley[2] found the drug to be effective in controlling the general symptoms of mastitis but made no mention as to whether the Staphylococcus organism had been eliminated from the milk. Simon and Schmidt[9] found that treatment of streptococcal udder infections with 200 mg. and with 400 mg. of oxytetracycline infusion ointment eliminated the infection from 60.0 and from 59.4 per cent of the quarters, respectively, without inflammatory response by the udder.

Tünsmeyer,[11] using three 400-mg. infusions of oxytetracycline ointment, reported that 80 per cent of the treated coliform mastitis was clinically improved and that 66 per cent became bacteriologically negative; also, that all of 15 streptococcal and four of five staphylococcal mastitis infections were eliminated by oxytetracycline infusions.

Barnes[1] treated 4 cows with bovine mastitis caused by *Klebsiella pneumoniae* with oxytetracycline. Systemic symptoms were relieved in each by intramuscular administration, but local treatment did not always eliminate the organism from the infected quarters.

Gratzl and Jaksch[5] found that the injection of oxytetracycline, 10 mg. per kilogram, in the horse frequently resulted in marked general weakness. The local inflammatory response was much more severe following subcutaneous than following intramuscular injections.

Morse, Spencer, and Simon[6] determined the *in vitro* sensitivity to oxytetracycline of bacteria isolated from animals. They found that 15 strains of streptococci and 14 strains of staphylococci were inhibited by 1 μg. per milliliter, while 3 μg. per milliliter was necessary to inhibit three other strains of staphylococci. One microgram per milliliter was lethal to all strains of *Streptococcus agalactiae* and three strains of *Staphylococcus aureus*, but five strains of *Streptococcus uberis* and one strain of *Staph. aureus* were killed only by concentrations greater than 100 μg. per milliliter and two strains of *Staph. aureus* were killed by 7 μg. per milliliter. Two strains of *Pseudomonas aeruginosa* of animal origin were inhibited *in vitro* by 50 μg. per milliliter for twenty-four hours. Two other strains were inhibited by 100 μg. per milliliter but were killed only by concentrations greater than 100 μg. per milliliter.

Richou, Gerbeaux, and Schlaepfer[8] studied the sensitivity to oxytetracycline of 21 strains of *Staph. aureus*, 16 of which were inhibited by 1.25 μg. per milliliter or less and five by between 1.25 and 5.0 μg. per milliliter.

Edwards[3] reported that several strains of *Str. agalactiae*, *Str. uberis*, and *Staph. aureus* were inhibited by 1.0 μg. per milliliter of oxytetracycline for twenty-four hours at 37 C. in broth.

DESCRIPTION OF THE HERD

All treatment of mastitis with oxytetracycline* was conducted in the Iowa State College dairy herd composed of approximately 120 milking cows. The five major dairy breeds were represened. Sanitary measures were above average. The udders were washed before the cows entered the milking stalls and wiped with an individual, clean, damp cloth immediately prior to milking. Each milking unit had two claw-type heads so that one could be sanitized by immersion in water at 170 F. for approximately four minutes while the other was in use. The cups were cooled with running tap water before being placed on the teats. Between milkings, twice daily, the teat cups were filled with a

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* Furnished by Chas. A. Pfizer Co., Brooklyn, N. Y.

1 per cent solution of commercial lye. A strip cup was available and any abnormalities observed during milking were reported to the author.

The entire herd was tested annually and was free of brucellosis and tuberculosis.

No mastitis caused by *Str. agalactiae* had been found in the herd since 1944.[7] The incidence of mastitis in 124 cows in 1952 was 44 cows (35.5 %) infected in one or more quarters. Eighty-one (16.5 %) of 491 quarters of those cows were infected as follows:

Staphylococcus aureus	45.78 %
Streptococcus uberis	42.16 %
Pseudomonas aeruginosa	7.22 %
Streptococcus dysgalactiae	2.41 %
Escherichia coli	2.41 %
	99.98 %

METHODS OF PROCEDURE

Diagnostic Methods.—Strip cup test, Hotis test, pH determination, catalase test, Whiteside test, and bacteriological culturing were used to establish a diagnosis because no one test was completely accurate nor did it furnish all the desired information.

Milk samples were incubated overnight at 37 C. and then streaked on one quarter of a 5 per cent bovine blood agar plate. The plates were incubated for sixteen to twenty hours and then examined for growth. Small colonies which resembled those of streptococci were picked into infusion broth and further differentiated by biochemical fermentations. Typical colonies showing double-zone hemolysis were identified as *Staph. aureus*. If the hemolysis was weak or atypical, a colony was inoculated onto 7.5 per cent sodium chloride, mannite agar and, later, a loopful was transferred for the coagulase test. Coagulation of dilute rabbit plasma within twenty-four hours was considered as confirmation of the presence of *Staph. aureus*. *Pseudomonas aeruginosa* was identified directly from the blood agar plate on the basis of *beta* hemolysis, green pigment formation, and the odor of trimethylamine. Colonies resembling those of coliform organisms were transferred into infusion broth and then tested for biochemical reactions. Many were further identified by their antigenic structure.

Whenever *Staph. aureus* or *Str. uberis* was identified from a milk sample, a diagnosis of mastitis was made. Coliform organisms and *Ps. aeruginosa* were not considered of pathogenic importance unless they were isolated from two or more successive samples and there were definite signs of inflammation with no other pathogenic organism being isolated.

Treatment Procedure.—Mastitis therapy was initiated at the time of the first examination. Usually one 400-mg. tube of commercial ointment infusion (7.1 Gm., 60 mg./Gm.) of oxytetracycline was used every twenty-four hours for three treatments. If the condition was unusually severe, 800 mg. was infused once daily usually for three days but for five or six days when the symptoms were not alleviated. Acute mastitis accompanied by systemic symptoms was treated by intramuscular administration of 1 or 2 Gm. of oxytetracycline, in addition to the local treatment, repeated every twelve or twenty-four hours until improvement was observed. No other medication was used.

Results were determined by periodic examination of milk samples with the same procedure as for diagnosis. The treatment was considered successful if the causal organism could not be isolated from the milk during any of three weekly examinations following treatment and if the indirect tests indicated that the inflammation was subsiding.

Procedure for Determining Irritant Properties.—Since observations had indicated that oxytetracycline was irritating to the mammary gland, the clinical response and the catalase and Whiteside reactions of a few locally treated quarters were studied. Milk samples of six quarters, three normal and three infected, were collected once daily for a week preceding treatment with oxytetracycline infusion ointment and continued until five days following the last infusion. Physical examinations, including palpation and strip cup tests, were also made.

Methods for Determining Sensitivity of Mastitis Organisms to Oxytetracycline.—To determine the *in vitro* sensitivity of *Staph. aureus* and *Str.*

TABLE 1—Comparison of the Results of Treatment of Clinical and Subclinical Cases of Mastitis with Oxytetracycline

Etiological agent	Type of case	No. treated	No. recovered	Recovered (%)	Over-all recovery (%)
Str. uberis	Clinical	12	9	75.00	82.69
	Subclinical	34	29	85.29	
Str. dysgalactiae	Clinical	3	1	33.30	33.30
	Subclinical	....	....	.....	
Staph. aureus	Clinical	15	2	13.33	25.00
	Subclinical	29	9	31.03	
Coliform group	Clinical	13	9	69.23	69.23
	Subclinical	....	....	.....	
Ps. aeruginosa	Clinical	8	0	0.00	0.00
	Subclinical	1	0	0.00	
Total	Clinical	51	21	41.17	
	Subclinical	64	38	59.37	
Grand total		115	59	51.30	51.30

uberis to oxytetracycline, strains were selected from quarters that had and that had not recovered with oxytetracycline therapy. Twenty-one strains were used, 12 of *Staph. aureus* and nine of *Str. uberis*.

TABLE 2—Correlation of Results of Treatment in Subclinical Cases of Mastitis with Duration of Infection

	Recently acquired infections			Long standing infections		
	Treated (No.)	Recovered (No.)	Recovered (%)	Treated (No.)	Recovered (No.)	Recovered (%)
Streptococcal	16	12	75.0	18	14	77.7
Staphylococcal	11	4	36.4	27	5	18.5

Bacteriostatic levels were determined by inoculating 24-hour broth cultures of the organisms onto 7.5 by 12.0 in. blood agar plates containing varying amounts of oxytetracycline per milliliter. The plates were incubated at 37 C. and examined for growth at twenty-four and forty-eight hours after inoculation. All 21 strains were tested simultaneously on five plates each containing one of the following antibiotic concentrations: 2.5, 1.25, 0.63, 0.32, or 0.16 μg. per milliliter.

The bactericidal concentration of oxytetracycline for each strain of *Staph. aureus* was determined by using serial doubling dilutions of antibiotic in infusion broth. The concentrations used were: 200, 100, 50, and 25 μg. per milliliter. Each tube was inoculated with 1 drop of a 10^{-3} dilution of a 24-hour broth culture of the Staphylococcus organism. Twenty-four and forty-eight hours following inoculation, a loopful of material from each dilution was streaked onto a quarter of a 5 per cent bovine blood agar plate and, if no growth appeared in twenty-four hours, the concentration of oxytetracycline represented by the sample was considered to be lethal.

The bactericidal concentration of oxytetracycline for each strain of *Str. uberis* was determined by the same procedure, except that the concentrations used were: 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, and 0.32 μg. per milliliter.

RESULTS

Treatment was most successful in cases caused by streptococci and coliform organisms (table 1). Only 25 per cent of the treated *Staph. aureus* infections and none of the *Ps. aeruginosa* infections were eliminated. The thirteen infections caused by the coliform group included the genera Escherichia, Klebsiella, Aerobacter and Paracolobactrum. The drug was more effective in subclinical infections, especially those of *Staph. aureus*. Totals for all cases showed treatment of subclinical infections to be 18.2 per cent more effective than treatment of clinical infections.

The results of treatment of recently acquired and long standing subclinical infections are compared (table 2) using an arbitrary time of one month to divide the two types. With staphylococcal mastitis recently acquired infections were successfully treated at a rate approximately twice that of long standing infections, while with streptococcal mastitis treatment had about the same efficacy in both types.

Irritant Properties of Oxytetracycline.—The inflammatory response to intramammary infusion of oxytetracycline ointment (table 3) in both normal and infected quarters varied. The quarters receiving one infusion showed less reaction with the catalase test than those receiving three, indicating that the inflammatory reaction of the mammary gland may be quantitative.

TABLE 3—Results of the Examination of Milk Samples from Three Infected and Three Non-infected Quarters Using Catalase and Whiteside Tests Before, During, and After Treatment with Oxytetracycline

No. daily doses (400 mg.)	Animal and quarter	Seven days preceding treatment			During treatment			Five days following last treatment			Results
		Whiteside	Catalase* Range	Catalase* Ave.	Whiteside	Catalase Range	Catalase Ave.	Whiteside	Catalase Range	Catalase Ave.	
1	3157 LF	±	3–8	4.7	+	13–17	15.6	—	3–12	6.6	Normal quarter
1	2392 LF	—	4–6	5.0	+	6–8	7.0	—	4–6	5.0	Normal quarter old cow
1	2643 RR	±	3–8	5.3	+	5–7	6.0	—	2–4	3.0	Chronic *Str. uberis* and *Staph. aureus* infection
3	3157 RR	—	1–5	3.0	+	20.0	20.0	±	7–16	10.2	Normal quarter
3	2392 LR	+	11–17	13.3	+	12–14	13.3	+	10–14	12.6	Chronic infection *Staph. aureus*
3	2643 RF	—	6–8	6.7	+	12–15	14.0	—	6–12	9.2	Chronic infection *Staph. aureus*

*All catalase values expressed as milliliters of free gas.

Most of the quarters returned to the same range of catalase values within five days after the last administration. The quarters receiving only one treatment became negative to the Whiteside test sooner than those receiving three.

Palpations conducted during this experiment showed that some quarters became inflamed so that mild swelling and edema could be detected. This was seen only in the quarters that showed a marked reaction to the laboratory tests. Palpation of other quarters showed only a mild sensitivity or no change.

Sensitivity of Mastitis Organisms to Oxytetracycline.—Twenty of 21 strains of *Staph. aureus* and *Str. uberis* were inhibited (table 4) for twenty-four hours by 1.0 μg. per milliliter, or less, of oxytetracycline. One strain of *Staph. aureus* was inhibited by 2.0 μg. per milliliter. Generally, the staphylococci were only slightly more resistant than the streptococci. In most instances, a higher concentration of drug was required to inhibit the organisms for forty-eight hours than for twenty-four hours. There is a striking difference in the concentrations necessary to kill strains of the two organisms, *Staph. aureus* being resistant to concentrations of at least 100 times those which were lethal to *Str. uberis*.

DISCUSSION

Although oxytetracycline did not prove to be as highly effective as might be hoped for, it has been shown to be of definite value in the treatment of infectious bovine mastitis. Local symptoms were relieved in all quarters, but when the infective agent was not eliminated, symptoms frequently reappeared after therapy was discontinued. Intramuscular administration of oxytetracycline was routinely successful in relieving the systemic symptoms which accompanied acute mastitis, irrespective of the etiological agent.

There is little published work with which to compare the results of this investigation, particularly on mastitis caused by *Staph. aureus* and *Ps. aeruginosa*. Some comparison might be made, however, with the results obtained by Simon and Schmidt [9] in the treatment of *Str. agalactiae* mastitis with one infusion of oxytetracycline. Their results were 23 per cent less favorable than those obtained in this work with three treatments. The *in vitro* sensitivity of *Str. agalactiae* and *Str. uberis* to oxytetracycline is very similar [3, 6] and, although *Str. uberis* seems to be more resistant to the lethal effect, the better results in this investigation might be attributed to the repeated doses.

The rate of recovery in *Staph. aureus* mastitis may be influenced by the relative resistance of the organism to the lethal effect of oxytetracycline or to the duration of the infection in the udder at the time of treatment. Both *Staph. aureus* and *Str. uberis* are inhibited *in vitro* by approximately 1 μg. per milliliter of the antibiotic. However, *Staph. aureus* is far more resistant to the lethal effect and if the organisms must be killed to eliminate infection from the mammary gland this refractiveness would strongly influence the recovery rate. This would indicate that large doses of oxytetracycline are necessary to eliminate udder staphylococcic infections. No information was obtained as to why the treatment of recently acquired subclinical *Staph. aureus* mastitis was approximately twice as successful as long-standing infections but the importance of early treatment of mastitis is emphasized.

Although the number was small, a significant percentage of the cows with coli-

TABLE 4—Bacteriostatic and Bactericidal Levels of Oxytetracycline in Vitro for 12 Strains of Mastitis Staphylococci and 9 Strains of Streptococci

Concentration in μg./ml.	Bacteriostatic levels				Bactericidal levels			
	Str. uberis		*Staph. aureus*		*Str. uberis*		*Staph. aureus*	
	24 (hr.)	48 (hr.)	24 (hr.)	48 (hr.)	24 (hr.)	48 (hr.)	24 (hr.)	48 (hr.)
<1.0	9*	3	11	0	....	....	....	....
1.0–2.0	0	5	1	5	....	....	....	....
2.0–3.0	0	1	0	7	....	....	....	....
<10	....	....	....	....	7	8	0	0
10–20	....	....	....	....	1	1	0	0
20–100	....	....	....	....	1	0	1	1
100–200	....	....	....	....	0	0	2	2
>200	....	....	....	....	0	0	9	9

* Number of strains.

form mastitis recovered with oxytetracycline therapy, which concurs with the report of Tünsmeyer.[11] There were too few of them to be considered by their genus. Intramuscular oxytetracycline, used to treat coliform mastitis cases which were accompanied by systemic symptoms, was routinely effective.

When oxytetracycline therapy relieved the local symptoms only temporarily another drug was used to eliminate the infective agent.[1]

The irritation study leaves little doubt that infusion of oxytetracycline ointment produces a measurable amount of inflammation. This is contrary to the work of Simon and Schmidt.[9] Certain clinical observations also showed oxytetracycline to be irritating. One quarter with a chronic staphylococcic infection was treated twice with 2-Gm. doses (5 commercial tubes) of oxytetracyline infusion ointment twenty-four hours apart. At the time of the second treatment the secretion was blood-tinged and the quarter was sensitive to mild pressure. Twenty-four hours after the second treatment, the secretions were obviously bloody and the quarter was edematous and inflamed. No other therapy was used and in a few days the quarter and its secretion gradually returned to the same condition as before the infusions. The infection was not eliminated.

Intramuscular administration of 1 or 2 Gm. of oxytetracycline usually caused no distress to the patient except temporary pain at the site of injection. However, an undesirable reaction in a 1,000-lb. cow with a chronic staphylococcic mastitis in one quarter followed two treatments with 400 mg. in an infusion ointment and 5 Gm. intramuscularly. No untoward response was noticed in the quarter at any time but twenty-four hours following the second daily dose, there were marked general symptoms of anxiety, restlessness, anorexia, and weakness; also, a distinct edema and soreness at the points of injection. Treatment was stopped but the cow did not return to normal for approximately five days. Gratzl and Jaksch [5] described a comparable weakness in horses.

SUMMARY AND CONCLUSIONS

1) During treatment of 115 cows with controlled clinical cases of bovine mastitis, oxytetracycline was found to be effective against various etiological agents in the following percentages: streptococci, 82.9, *Staphylococcus aureus*, 25.0, coliform organisms, 69.2, and *Pseudomonas aeruginosa*, 0.0.

2) Nearly all cows with acute cases showed clinical improvement during local therapy whether or not the treatment was successful.

3) Results were better with subclinical than with clinical cases of mastitis.

4) Results were better in animals with recently acquired than with long-standing mastitis, especially in mastitis caused by *Staph. aureus*.

5) Strains of *Staph. aureus* from cases of mastitis were relatively resistant to the lethal effect of oxytetracycline, which might in part explain the refractiveness of these infections to therapy.

6) Use of oxytetracycline intramuscularly aided in the relief of systemic symptoms in all animals, irrespective of the etiological agent.

7) Oxytetracycline was found to be irritating in certain instances when administered both intramuscularly and intramammarily.

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Bovine Antibiotic Blood Levels Obtained with Antibiotics in Oil Suspensions

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THE PROLONGATION of antibiotic blood levels from a single dosage is of particular value in veterinary medicine since repeated injections may be not only inconvenient and uneconomic but also impractical because of environmental circumstances.

The chemistry and pharmacology of a repository form of penicillin, *N,N'*-dibenzylethylenediamine dipenicillin G (benzathine penicillin G), was described in 1951[1-3] and penicillin blood levels lasting for two weeks or longer after a single intramuscular injection of this compound in human beings were reported in 1952.[4] Veterinary literature has included information on the duration of blood levels produced by a single intramuscular injection of this salt in cattle,[5] sheep, swine,[6] dogs,[7] and turkeys.[8] In general, comparison of benzathine and procaine penicillin G indicates that the penicillemia produced by benzathine is of a lower level but that it is prolonged over a period of many days.

Since all of the reported information on benzathine involved its aqueous suspension, this study was undertaken to determine the difference in effect, if any, produced by oil suspensions.

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MATERIALS AND METHODS

Blood serum samples from the first group of 10 normal, adult, milking cattle were examined immediately prior to injection and found to be negative for any bacteriostatic activity. These animals were then given a single intramuscular injection of 2,000,000 units of benzathine in sesame oil with 2 per cent aluminum monostearate (volume, 4 cc.). Blood samples were withdrawn at the postinjection times indicated in table 1 and the serum content of penicillin was determined by the microbiological cup-plate method using *Sarcina lutea* as the test organism. The results are listed in table 1.

After similar preinjection serum examinations, a second group of 10 normal, adult, milking cattle were given a single intramuscular injection of 2,000,000 units of benzathine and 2,000,000 units of procaine penicillin G in sesame oil with 2 per cent aluminum monostearate (volume, 8 cc.). Blood samples were withdrawn at the postinjection times indicated and the serum content of penicillin determined by the microbiological cup-plate method using *S. lutea* as the test organism. The results are listed in table 2.

Additionally, the majority of the reports in the literature concerning streptomycin and dihydrostreptomycin relate to aqueous suspensions.[9-11] Therefore, after the usual preinjection serum examinations, a third group of 10 normal, adult, milking cattle received 600,000 units of benza-

TABLE 1—Bovine Blood Serum Levels (Including Milk Levels) After Injections of Dibenzylethylenediamine Dipenicillin G in Sesame Oil with 2 Per Cent Aluminum Monostearate*

Cow No.	Cow wt. (lb.)	Units of penicillin/cc. of blood serum												Units of penicillin/cc. of milk	
		Normal	1 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.	168 hr.	192 hr.	216 hr.	6 hr.	72 hr.
1	650	0	0.0	0.017	0.019	0.024	0.026	0.0	0.0	0.020	0.016	0.022	0.020	0	0
2	1,075	0	0.127	0.167	0.108	0.081	0.045	0.029	0.023	0.020	0.0	0.0	.....	0.0	0
3	500	0	0.0	0.0	0.073	0.030	0.024	0.045	0.022	0.036	0.016	0.016	0.015	0	0
4	825	0	0.0	0.0	0.110	0.019	0.020	0.027	0.014	0.022	0.014	+	+	0	0
5	825	0	0.016	0.012	0.0	0.020	0.017	0.020	0.0	0.016	0.0	0.016	0.017	0	0
6	825	0	0.025	0.015	0.013	0.020	0.024	0.032	0.021	0.018	0.0	0.012	0.012	0	0
7	775	0	0.0	0.0	0.0	0.020	0.020	0.026	0.022	0.026	0.013	0.012	+	0	0
8	825	0	0.0	0.0	0.0	0.029	0.040	0.031	0.018	0.020	0.0	0.0	0.0	0	0
9	825	0	0.0	0.0	0.0	0.031	0.030	0.033	0.027	0.027	0.015	0.015	0.0	0	0
10	870	0	0.0	0.0	0.019	0.022	0.024	0.026	0.018	0.021	0.0	0.0	0.0	0	0
Average		0	0.004	0.004	0.025	0.023	0.022	0.024	0.014	0.023	0.007	0.012	0.010	0	0

* Dibenzylethylenediamine dipenicillin G—500,000 units/cc.; intramuscular injection—single dose of 2,000,000 units (4 cc.).

+ = Detectable but unmeasurable activity.

TABLE 2—Bovine Blood Serum Levels (Including Milk Levels) After Injections of Dibenzylethylenediamine Dipenicillin G and Procaine Penicillin G in Sesame Oil with 2 Per Cent Aluminum Monostearate*

Cow No.	Cow wt. (lb.)	Normal	Units of penicillin/cc. of blood serum 1 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.	168 hr.	192 hr.	216 hr.	Units of penicillin/cc. of milk 6 hr.	72 hr.
11	810	0	0.141	0.172	0.053	0.058	0.050	0.032	0.023	0.023	0.0	0.0	.....	0.0	0
12	810	0	0.127	0.167	0.108	0.081	0.045	0.029	0.023	0.020	0.0	0.0	.....	0.0	0
13	790	0	0.181	0.157	0.058	0.053	0.047	0.032	0.020	0.026	0.0	0.0	.....	0.0	0
14	800	0	0.144	0.162	0.052	0.045	0.034	0.021	0.019	0.016	0.0	0.0	.....	0.0	0
15	1,075	0	0.270	0.142	0.045	0.046	0.030	0.022	0.017	0.018	0.0	0.0	.....	0.013	0
16	880	0	0.157	0.108	0.057	0.058	0.040	0.032	0.017	0.018	0.0	0.0	.....	0.0	0
17	925	0	0.177	0.175	0.083	0.081	0.050	0.040	0.019	0.020	0.0	0.0	.....	0.0	0
18	900	0	0.162	0.185	0.066	0.058	0.038	0.032	0.016	0.016	0.0	0.0	.....	0.4	0
19	850	0	0.181	0.180	0.073	0.062	0.048	0.030	0.028	0.014	0.012	0.014	0.016	0.014	0
20	875	0	0.149	0.114	0.070	0.056	0.048	0.030	0.018	0.017	0.0	0.0	.....	0.013	0
Average		0	0.169	0.156	0.066	0.060	0.043	0.030	0.020	0.019	0.001	0.001	.....	0.004	0

* Dibenzylethylenediamine dipenicillin G—250,000 units/cc.; procaine penicillin G—250,000 units/cc.; intramuscular injection—single dose of 4,000,000 units (8 cc.).

TABLE 3—Bovine Blood Serum Levels (Including Milk Levels) After Injections of Dibenzylethylenediamine Dipenicillin G, Procaine Penicillin G, and Streptomycin Sulfate in Sesame Oil with 2 Per Cent Aluminum Monostearate*

Cow No.	Cow wt. (lb.)	Normal	Micrograms of streptomycin/cc. of blood serum 1 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.	Micrograms of streptomycin/cc. of milk 6 hr.	72 hr.
21	890	0	3.480	3.800	0.360	0.0	0	0	0.018	0
22	850	0	4.300	4.800	0.158	0.0	0	0	0.023	0
23	875	0	4.300	3.500	0.136	0.0	0	0	+	0
24	825	0	3.200	3.800	0.350	0.118	0	0	0.0	0
25	900	0	2.800	1.660	0.0	0.0	0	0	0.0	0
26	800	0	3.480	3.100	0.108	0.0	0	0	0.0	0
27	825	0	3.480	3.380	+	0.0	0	0	0.0	0
28	870	0	4.700	3.500	0.330	0.0	0	0	0.108	0
29	880	0	1.650	0.320	0.330	0.0	0	0	0.0	0
30	900	0	2.800	0.290	0.102	0.0	0	0	0.0	0
Average		0	3.419	2.815	2.082	0.012	0	0	0.017	0

* Dibenzylethylenediamine dipenicillin G—50,000 units/cc.; procaine penicillin G—150,000 units/cc.; streptomycin sulfate—250 μg. base/cc.; intramuscular injection—single dose of 2,400,000 units of combined penicillins and 3 Gm. base of streptomycin sulfate (12 cc.).

+ = Detectable but unmeasurable activity.

TABLE 4—Bovine Blood Serum Levels (Including Milk Levels) After Injections of Dibenzylethylenediamine Dipenicillin G, Procaine Penicillin G, and Streptomycin Sulfate in Sesame Oil with 2 Per Cent Aluminum Monostearate*

Cow No.	Cow wt. (lb.)	Normal	Units of penicillin/cc. of blood serum 1 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.	Units of penicillin/cc. of milk 6 hr.	72 hr.
21	890	0	0.162	0.102	0.056	0.026	0.0	0	0.0	0
22	850	0	0.260	0.198	0.016	0.016	0.0	0	0.033	0
23	875	0	0.162	0.170	0.033	0.0	0.0	0	0.019	0
24	825	0	0.178	0.210	0.062	0.148	0.0	0	0.0	0
25	900	0	0.168	0.053	0.013	0.0	0.0	0	0.0	0
26	800	0	0.178	0.160	0.032	0.020	0.015	0	0.023	0
27	825	0	0.133	0.079	0.020	0.0	0.0	0	0.017	0
28	870	0	0.133	0.215	0.365	0.0	0.0	0	0.053	0
29	880	0	0.140	0.180	0.047	0.020	0.0	0	0.0	0
30	900	0	0.168	0.176	0.039	0.0	0.0	0	+	0
Average		0	0.168	0.154	0.068	0.023	0.001	0	0.016	0

* Dibenzylethylenediamine dipenicillin G—50,000 units/cc.; procaine penicillin G—150,000 units/cc.; streptomycin sulfate—250 μg. base/cc.; intramuscular injection—single dose of 2,400,000 units of combined penicillins and 3 Gm. base of streptomycin sulfate (12 cc.).

+ = Detectable but unmeasurable activity.

thine, 1,800,000 units of procaine penicillin G, and 3 Gm. of streptomycin base as sulfate (volume, 12 cc.) (formula not prepared specifically for this study). Blood samples were withdrawn at the postinjection times indicated and the serum content of both penicillin and streptomycin were determined by the microbiological cup-plate method using *S. lutea* as the test organism for penicillin and *Bacillus subtilis* as the test organism for streptomycin. The results are listed in tables 3 and 4.

CONCLUSIONS AND SUMMARY

In each of the above groups of cattle, examinations of the milk were made at the interval specified in the tables to determine the antibiotic concentrations in this secretion. No significant bacteriological change was found in any of the milk samples for sixty days after the parenteral injection.

The many variables involved in a project of this type and evaluation by a biological assay make definite conclusions difficult. However, after a study of the literature, consideration of our past experiences, and examination of the data presented here, it is believed that, in comparison with previous reports on aqueous suspensions:

1) Benzathine in sesame oil with 2 per cent aluminum monostearate produces much lower penicillin blood levels during the first twenty-four hours after injection. After this time, the penicillin concentrations may be slightly higher but they are prolonged for six to nine days, the identical interval produced by the aqueous suspension. Individual variations are not unusual.

2) Benzathine and procaine penicillin G in sesame oil with 2 per cent aluminum monostearate produce somewhat lower penicillin blood levels during the first twenty-four hours after injection. After this time, the penicillin concentrations may be slightly higher, with a larger percentage of the test animals demonstrating penicillin blood concentration for a longer period but, again, the effective duration interval is six days, similar to that reported for the aqueous suspension.

3) Streptomycin sulfate administered in sesame oil with 2 per cent aluminum monostearate produces streptomycin blood concentrations of greater duration than do aqueous suspensions. Even in slightly smaller doses, an effective level was maintained for twenty-four hours compared with twelve to eighteen hours produced by aqueous suspensions.

4) After parenteral administration of usual doses, small amounts of penicillin and streptomycin excreted in the milk showed no direct relationship to the blood concentrations.

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In Vitro Inhibition of Mixed Strains of *Cryptococcus Neoformans* Isolated from Cattle

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IN A PREVIOUS paper, Simon *et al.*[1] described an outbreak of bovine cryptococcosis in which approximately 50 of 280 lactating animals had been affected. *Cryptococcus neoformans* was cultured from the milk of 10 animals which were clinically ill or had elevated temperatures.

Since treatment of the cryptococcal infection in the cattle was ineffective, an *in vitro* evaluation of a variety of materials with known antimycotic activity was undertaken. Because of the potential hazard of cryptococcal infections in human beings, a number of common laboratory disinfectant solutions was also tested for cryptococcicidal activity.

MATERIALS AND METHODS

The bovine strains of *C. neoformans* which were used as the test organisms were obtained from milk or tissue from animals involved in the outbreak described (Simon *et al.*,[1] 1953) as follows: 531N—isolated from the supramammary lymph node of cow 531; 531U—isolated from the milk of cow 531; 230—isolated from mammary tissue of cow 230; 268D—isolated from the milk of cow 268; and guinea pig spleen—obtained from a guinea pig which had been inoculated intraperitoneally with 0.1 ml. of a saline suspension of 531N with a light transmission of 67 per cent, Coleman nepho-colorimeter 655-mμ filter.

Experiment 1.—Sabouraud's dextrose-agar slopes were streaked with the respective strains of *C. neoformans* from stock cultures and incubated at 37 C. for forty-eight hours. Growth was washed from the slopes with a sterile saline solution and the washings were pooled. The suspension was adjusted to 70 per cent light transmission as determined by a Coleman nepho-colorimeter, model 9, with a 655-mμ filter. One tenth milliliter of this suspension was inoculated into 19- by 150-mm. cuvettes, each of which contained 9 ml. of sterile Sabouraud's dextrose broth. The antibiotics tested for inhibitory activity against *C. neoformans* were dissolved in sterile saline and aqueous solutions of paraben (*n*-propyl-*p*-hydroxybenzoate), metaben (methyl *p*-hydroxybenzoate), and ethyl vanillate (ethyl 4-hydroxy-3-methoxy-benzoate) and were sterilized by autoclaving at 15 lb. for twenty minutes. Suitable dilutions were made so that 1 ml. added to the broth would give the desired final concentration.

TABLE 1—Effect of Potassium Penicillin and Dihydrostreptomycin on Mixed Suspensions of Cryptococcus Neoformans (Percentage of Light Transmission Observed at Twenty-four, Forty-eight, Seventy-two, and Ninety-six Hours Following Incubation at 37 C.)*

Material	Concentration (units/ml.)	Light transmission (%) at hours: 24†	48	72	96
Control	.....	....	90	84	79
Penicillin, potassium	10,000	....	93	91	87
	5,000	....	92	90	86
	2,500	....	92	88	85
	2,000	....	89	87	82
	1,000	....	88	86	82
	500	....	86	84	81
	250	....	88	85	80
Dihydrostreptomycin	10,000	....	98	99	94
	1,000	....	93	88	83
	500	....	92	90	84
	100	....	93	87	84
	50	....	91	88	93
	10	....	91	87	83

* Subcultures were not made as growth was evident grossly in all tubes at ninety-six hours. † Colorimeter was not functioning at 24-hour reading.

Light-transmission readings were taken at twenty-four, forty-eight, seventy-two, and ninety-six hours following inoculation. The figures given in the tables represent the average of duplicate tubes of each material. One tenth milliliter from each tube which showed little or no turbidity was subcultured on Sabouraud's dextrose agar to detect growth.

Because a limited number of cuvettes was available, this report consists of a number of trials.

Experiment 2.—The strains of *C. neoformans* used in experiment 1 were employed in experiment 2; however, the inoculum was much heavier, the light transmission being approximately 10 per cent. One tenth milliliter of this heavy mixed suspension of *C. neoformans* was added to 9.9 ml. of the test material, thoroughly mixed, and incubated at 37 C. Aliquots of 0.1 ml. were removed at fifteen, thirty, sixty, 120, and 180 minutes and cul-

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TABLE 2—Inhibitory Effect of Polymyxin B, Neomycin Sulfate, Terramycin Hydrochloride, and Bacitracin on Mixed Suspensions of Cryptococcus Neoformans (Percentage Light Transmission Observed at Twenty-four, Forty-eight, Seventy-two, and Ninety-six Hours Following Incubation at 37 C.)

Material	Concentration (units/ml.)	Light transmission (%) at hours: 24	48*	72	96	Subculture
Control	.....	98	.....	90	81	+++
Polymyxin B (aerosporin)	500	99	.....	99	98	—
	250	98	.....	97	96	—
	125	99	.....	98	96	—
	100	100	.....	98	98	—
	50	100	.....	99	98	—
	25	99	.....	98	98	—
Neomycin sulfate†	500	99	.....	100	100	—
	250	100	.....	100	100	—
	125	99	.....	100	98	—
	100	99	.....	99	99	—
	50	98	.....	99	99	—
	25	99	.....	99	98	—
Terramycin hydrochloride†	500	(too turbid to be read colorimetrically with available filter)				+++
	250					+++
	125					+++
	100					+++
Bacitracin	900	92	.....	74	67	+++
	450	93	.....	68	63	+++
	225	85	.....	67	63	+++

* Colorimeter was not functioning at 48-hour reading. † Five tenths of a gram of the respective antibiotics was considered equivalent to 500,000 units and all dilutions were made on this basis.

tured on Sabouraud's dextrose-agar slopes. The slopes were incubated at 37 C. and examined at twenty-four, forty-eight, seventy-two, and ninety-six hours for growth.

RESULTS AND DISCUSSION

Experiment 1.—High concentrations of potassium penicillin and dihydrostreptomycin showed only a slight inhibitory effect for mixed suspensions of bovine strains of *C. neoformans* (table 1).

Polymyxin B (aerosporin®) and neomycin sulfate, even when diluted to 25 units per milliliter, were lethal for mixed suspensions of *C. neoformans* (table 2), while

TABLE 3—Inhibitory Effect of Paraben, Metaben, Penicillin O, and Ethyl Vanillate on Mixed Suspensions of Cryptococcus Neoformans (Percentage Light Transmission Observed Twenty-four, Forty-eight, Seventy-two, and Ninety-six Hours Following Incubation at 37 C.).

Material	Concentration/ml.	Light transmission (%) at hours: 24	48	72	96	Subculture
Control	.....	93	89	84	80	+++
Paraben*	2.5 mg.	98	99	98	99	—
	1.25 mg.	98	98	96	97	—
	0.625 mg.	97	97	96	97	—
	0.312 mg.	98	98	96	97	—
	0.0156 mg.	97	97	95	97	—
Metaben†	2.5 mg.	98	97	96	96	—
	1.25 mg.	98	97	97	98	—
	0.625 mg.	98	97	96	98	+
	0.312 mg.	97	97	93	94	+++
	0.0156 mg.	97	95	93	91	+++
Penicillin O‡	4,000 units	95	90	86	84	+++
	2,000 units	95	90	86	85	+++
	1,000 units	94	91	87	85	+++
	500 units	95	90	85	84	+++
Ethyl vanillate§	3.0 mg.	98	97	96	96	—
	2.5 mg.	99	98	97	97	—
	2.0 mg.	100	98	97	97	—
	1.5 mg.	100	98	97	98	—
	1.0 mg.	100	98	97	98	—
	0.75 mg.	100	99	98	99	—
	0.50 mg.	100	98	97	97	+
	0.25 mg.	100	98	96	94	+++

* *n*-propyl *p*-hydroxybenzoate; † methyl *p*-hydroxybenzoate; ‡ furnished through the courtesy of the Upjohn Company, Kalamazoo, Mich.; § furnished through the courtesy of the Medical Division of E. R. Squibb and Sons, New York, N. Y.

TABLE 4—Cryptococcicidal Effect of Common Laboratory Materials (0.1-ml. Aliquots Taken at Fifteen, Thirty, Sixty, Ninety, and 180 Minutes Following Exposure and Examined at Twenty-four, Forty-Eight, Seventy-two, and Ninety-six Hours Incubation at 37 C.)

Material	Incubation 24 hours					Incubation 48 hours					Incubation 72 hours					Incubation 96 hours				
	Time aliquot taken:					Time aliquot taken:					Time aliquot taken:					Time aliquot taken:				
	15	30	60	90	180	15	30	60	90	180	15	30	60	90	180	15	30	60	90	180
Saline control	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Roccal concentrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Furacin solution	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2O_2, 3%	++	++	++	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
H_2O_2, 30%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cresol, 3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl alcohol, 70%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl alcohol, 95%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 N,NaOH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acriflavine, 1:1,000 (aqueous)	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

no inhibition was manifested by terramycin® hydrochloride or bacitracin.

Paraben and ethyl vanillate were lethal for mixed suspensions of *C. neoformans* (table 3), while metaben exhibited a similar activity in concentrations of 1.25 mg. per milliliter. A lower concentration was somewhat inhibitory. Since paraben and metaben are relatively insoluble in water, their potential as therapeutic agents might be limited. Penicillin O possessed no antimycotic activity.

A few animals [1] were treated unsuccessfully with neomycin sulfate by the intramammary route. Aerosporin was not used. It is believed that the granulomatous reaction of the mammary gland in bovine cryptococcosis presents a physical barrier to the activity of therapeutic agents. No significance can be attached to a few empiric field therapy trials.

Experiment 2.—The cryptococcicidal effect of a number of common laboratory materials is summarized in table 4. Undiluted commercial roccal® (10 %), 3 per cent H_2O_2, 30 per cent H_2O_2, 3 per cent cresol, 70 per cent ethyl alcohol, 95 per cent ethyl alcohol, and 1 normal sodium hydroxide (1 N,NaOH) were cryptococcicidal when in contact with heavy mixed suspensions of *C. neoformans.* These materials should provide adequate laboratory disinfection with an exposure period of fifteen minutes.

SUMMARY

1) Ethyl vanillate (ethyl 4-hydroxy-3-methoxy-benzoate), paraben (*n*-propyl-*p*-hydroxybenzoate), metaben (methyl *p*-hydroxybenzoate), polymyxin B (aerosporin®), and neomycin sulfate were inhibitory for *Cryptococcus neoformans in vitro.*

2) Ten per cent roccal,® 3 per cent hydrogen peroxide, 30 per cent hydrogen peroxide, 3 per cent cresol, 70 per cent alcohol, 95 per cent alcohol, and 1 normal, sodium hydroxide (1 N,NaOH) exhibited a cryptococcicidal effect when in contact with mixed suspensions of *C. neoformans* for fifteen minutes.

The Enteric Distribution of 5-Hydroxytryptamine (Enteramine, Serotonin) in Some Large Domestic Mammals and the Appearance of 5-Hydroxytryptamine and the Enterochromaffin Cell System in the Embryonic Calf

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IT HAS BEEN observed by Erspamer [11,12] that 5-hydroxytryptamine (enteramine, serotonin) appears to be the secretion or deposit product of the enterochromaffin cell system, a peculiar cellular system present in vertebrates and in some invertebrate groups.

The cells of the enterochromaffin system are characterized by particular histochemical properties (chromaffinity, argentaffinity, ability to couple with diazonium salts, fluorescence in Wood's light, etc.) and by their histological fixation with formol and formol-containing fixatives.

So far, 5-hydroxytryptamine (briefly, 5-HT) has been demonstrated by chemical means or, more frequently, by paper chromatography or bio-assay, in the gastrointestinal tract of vertebrates [2] and ascidians, in the skin of amphibians, in the posterior salivary glands of octopods, and the hypobranchial body of some purple-producing mollusks, as well as in the spleen and blood serum of vertebrates.

According to recent investigations by Amin *et al.*,[1] Florey and Florey,[14] and Welsh,[20] certain central and peripheral nervous structures of vertebrates and invertebrates are also to be listed among the biological material containing 5-HT.

The main pharmacological actions of 5-HT may be summarized as follows:

a) Powerful stimulant action on the atropinized estrous uterus of the rat (threshold dose: 1 to 10^{6}–10^{9}). The sensitivity and the high specificity of the response of uterine smooth muscle to 5-HT accounts for the preference given to this organ in the biological titration of 5-HT.

b) Stimulant action on the canine urinary bladder, both isolated and *in situ*.

c) Remarkable positive inotropic, chronotropic, and tonotropic effects on the heart and pulsatile arteries of mollusks.

d) Conspicuous antidiuretic effect in rats; 5-HT seems to act on the contractile structures of the afferent vascular bed of the glomerulus, causing their spasm.

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On the basis of his experimental data, Erspamer [7–12] considers 5-HT as a true hormone influencing the control of intrarenal hemodynamics and, as a consequence, the water metabolism of the organism.

The aim of the present investigation is:

1) To determine the occurrence and distribution of 5-HT in the gastrointestinal tract of some large domestic mammals.

2) To investigate the interdependence between the first appearance of the enterochromaffin cells in the gastrointestinal mucosa of the bovine fetus and the first appearance of 5-HT in extracts of fetal intestine.

MATERIALS AND METHODS

The biological materials (portions of gastrointestinal tract and spleen tissue) were removed at the slaughterhouse immediately after the animals were killed. The intestine of adult animals was opened, rapidly washed and dried with filter paper, then treated, as were the other tissues, with three parts (weight/volume) of acetone. After standing for twenty-four to forty-eight hours at room temperature, the extracts were filtered and the solvent evaporated under reduced pressure. The remaining aqueous liquid was then brought to the desired volume either by further concentration or by dilution with saline. The bio-assay of the extracts was always carried out on the isolated uterus of the rat in estrus, using as a standard a solution of synthetic 5-HT picrate "farmitalia."

For the differentiation of 5-HT from other possibly disturbing uterus-stimulant substances, dibenamine was routinely used, in doses of 0.1 to 1.0 μg. per milliliter of tissue bath fluid. Both Erspamer [9] and Gaddum and Hameed [15] have shown that this sympatholytic drug irreversibly blocks the oxytocic action of 5-HT, while leaving unaffected that of acetylcholine, extracts of the posterior pituitary substance P, angiotonin, and other biogenic substances.

RESULTS

The 5-HT content of the different portions of the gastrointestinal tube is shown in table 1.

From the tabulated data the following conclusions may be drawn:

1) In ruminants 5-HT is present only in the fourth stomach, while absent in the others.

In the horse stomach the presence of the substance is doubtful; in that of the pig, 5-HT is present in detectable amounts.

2) The entire intestinal tract of all animals tested contains remarkable quantities of 5-HT. As a rule, the substance is particularly abundant at the beginning and at the end of the intestinal tube, *i.e.*, in the duodenum and in the distal portion of the colon. In some cases, *viz.* the horse and the calf, this latter portion may be even richer in this substance than the duodenum. With the exception of the calf, the intermediate intestine contains smaller amounts of 5-HT.

3) The above data on the distribution of 5-HT in the intestine of the large domestic animals are in agreement with the data reported for other mammals and are also in agreement with the available data on the quantitative distribution of the enterochromaffin cells in the mucosa of the different intestinal segments.

The data concerning the appearance and content of 5-HT in the intestine of a calf during embryonic development are shown in table 2.

Upon histological examination of sections of intestinal mucosa stained by the Masson-Hamperl method, the first typical enterochromaffin cells may be found as early as the fifth or sixth week of intrauterine life. The number of cells is extremely small and the specific argentaffin granules are rather rare.

During the following weeks both the number of cells and their richness in granules grows rapidly, attaining a maximum around the eleventh to the thirteenth week. From this stage on, apparently, the number of cells per gram of mucosa remains constant.

Upon examination of the foregoing data,

TABLE 2—The Appearance and Content of 5-Hydroxytryptamine in the Intestine of a Calf During Embryonic Development

Age of bovine fetus (days)	No. of anim.	Contents of enteramine free base (μg.) per Gm. of tissue	
		Intestine	Spleen
Up to 30 days	6	trace	no spleen
30 to 60	5	trace	no spleen
60 to 70	4	0.35	(or traces) traces of the organ
70 to 80	9	0.70	3.0
80 to 90	10	0.72	3.3
90 to 100	3	0.70	3.3
100 to 120	9	0.65	3.1
120 to 150	4	0.70	3.4
150 to 180	4	0.70	3.6
180 to 220	3	0.52	3.4

it seems reasonable to make the following assumptions:

1) The appearance of the first enterochromaffin cells coincides with the appearance of the first traces of 5-HT in extracts of the intestinal tissue.

As the number of the cells increases, following the embryonic development, there is a parallel increase in the 5-HT content of the intestinal extracts, and the relationship between the number of enterochromaffin cells and the 5-HT content in the intestinal wall remains constant throughout the entire intrauterine life.

2) 5-HT is to be found in the spleen immediately after the formation of the organ, *i.e.*, in the first few milligrams of tissue sufficient for a bio-assay.

The relative content of 5-HT in the spleen, *viz.* the content per gram of fresh tissue, seems to remain unchanged during the entire fetal life.

TABLE 1—The 5-Hydroxytryptamine Content of the Different Portions of the Gastrointestinal Tube

Animal species	Content of Enteramine as Free Base (μg./Gm.)							
	Stomach				Intestine			
	Rumen	Reticulum	Omasum	Abomasum	Small intestine (proximal)	Small intestine (distal)	Proximal colon	Distal colon
Adult bull	0	0	0	1.60	5.10	1.60	1.20	4.90
Calf	0	0	?	1.20	3.60	3.00	3.70	4.50
Sheep	0	0	?	3.20	4.10	2.00	2.50	2.90
Pig	0.50				4.00	0.50	0.28	0.53
Adult horse	?				0.72	0.32	0.49	1.00
Foal	?				0.74	0.45	0.74	0.98

DISCUSSION

It has been determined by Erspamer [13] that the enterochromaffin cells give, after formol fixation, exactly the same color reactions (chromaffin and other oxidation reactions, argentaffin reaction, coupling reaction with diazonium salts in alkaline medium, fluorescence in Wood's light, etc.) as do chromatographic spots of pure 5-HT pretreated with formol.

There exists a strict interdependence between the number of enterochromaffin cells in the gastrointestinal mucosa and the content of 5-HT in this mucosa, throughout the entire vertebrate series as shown by a number of workers.[5, 7, 9–12, 20] The intestinal mucosa of Teleostei and Cyclostomata, which lacks enterochromaffin cells,[5, 21] is also lacking in 5-HT.[8, 11, 12]

The same interdependence holds true for the posterior salivary glands of octopods, the hypobranchial body of prosobranchiate mollusks and the skin of Amphibia: 5-HT is present only in those species whose tissues contain chromaffin cells or a chromaffin secretion, as Erspamer points out.[11, 12]

Intestinal argentaffin carcinoids, which are tumors of typical enterochromaffin cells, may contain very large amounts of 5-HT (up to 2.5 mg. of 5-HT per Gm. of fresh tissue, according to Lembeck [16, 17]), and when carcinoids show diffuse metastases (Waldenström's "carcinoidosis"), the 5-HT serum level may raise in man from 0.1 μg./ml. to 4–6 μg./ml.[19]

The researches reported in this paper present further evidence in support of the hypothesis that intestinal 5-HT originates from the enterochromaffin cells of the gastrointestinal mucosa. The strict interdependence between the first appearance and number of enterochromaffin cells on the one hand, and the first appearance and abundance of 5-HT in intestinal extracts on the other, makes alternate explanations difficult.

Another point to be stressed here is that 5-HT circulates in the blood from at least the tenth to twelfth week. This is demonstrated by the presence of the substance in the spleen tissue. It seems probable also that in early embryonic life the platelets are the carriers of blood 5-HT and that spleen 5-HT is included in both the intact and in the disintegrating platelets.

The alternate hypothesis that 5-HT may be produced in the spleen itself does not appear tenable on the basis of the following observations:

1) Spleen tissue does not yet exist in the bovine fetus at the time of the first appearance of the enterochromaffin cells in the gastrointestinal mucosa and the detection of 5-HT in intestinal extracts.

2) Ascidians *(Cyona intestinalis)* do not contain thrombocytes in their hemolymph, nor do they possess any organ analogous to spleen; they show, however, typical enterochromaffin cells in their gastrointestinal mucosa, yet both Erspamer [8] and Lison [15] have shown that 5-HT can be readily found in their intestinal extracts.

3) Splenectomy in the experimental animal (rat) does not alter the number of the enterochromaffin cells in the gastrointestinal mucosa according to Ceriotti,[4] while Bigelow [3] has determined that splenectomy in human beings suffering from thrombocytopenic diseases restores to normal not only the low platelet count but also the very low blood 5-HT level.

4) 5-Hydroxytryptophan, the immediate precursor of 5-HT, has not yet been described in spleen tissue, whereas it has been found by Udenfriend (cited by Erspamer [11]) in extracts of gastric mucosa, together with 5-hydroxytryptophan decarboxylase, an enzyme which catalyzes the transformation of 5-hydroxytryptophan into 5-HT.[6]

SUMMARY

In the first part of this investigation the occurrence and distribution of 5-hydroxytryptamine (5-HT) in the gastrointestinal tract of some large domestic mammals is studied.

The latter experiments reported here have established the time of the first appearance of the enterochromaffin cells in the intestinal mucosa of the bovine fetus and the time of the first appearance of 5-HT in embryonal intestinal extracts. The results obtained appear to confirm the thesis that 5-HT is the secretion or deposit product of the enterochromaffin cells and demonstrate that the substance circulates in fetal blood at least as early as in the tenth to twelfth week of intrauterine life.

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The Course of Infection and Pathogenic Effect of *Trichostrongylus Axei* in Calves

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Trichostrongylus axei is a common and widely distributed nematode parasite of ruminants and of the equine species and, although its common habitat is the abomasum of ruminants and the stomach of horses, it may on occasion be recovered from the small intestine of these hosts. Numerous case reports have indicated that this small hairworm is the primary or contributory cause of clinical symptoms and death losses in sheep and goats. Experimental proof of the pathogenic effect of *T. axei* in pure or nearly pure infections in sheep has been recorded by Gibson [5-7] and Turner and Kates;[13] a profuse and protracted diarrhea and consequent dehydration of the host, frequently terminating in death, was typically produced. Reports of natural heavy infections in cattle, with death attributed to this parasite, have been made by M'Fadyean,[8] Baker,[3] Taylor,[12] Stewart and Crofton,[11] Corrie,[4] Schwartz,[10] and Andrews, Sippel, and Jones.[2] Since experimental proof of its pathogenicity in cattle is lacking, the experiment here reported was performed to determine the effects of this parasite on the health of calves 3 to 6 months old.

Procedure

Animals, Housing, and Diet.—Fourteen male, Holstein-Friesian calves, obtained from farms and dairies in the vicinity of Beltsville, Md., when less than 4 weeks old, were used in this experiment. Each calf was placed in a separate concrete-floored pen in a barn and was fed milk until about 2 months old. After weaning, the calves were fed U. S. No. 2 alfalfa hay and mixed grain, daily, in amounts adequate to insure good weight gains. Any uneaten hay or grain was weighed and the amount consumed recorded.

All pens were cleaned daily and washed and scrubbed thoroughly with hot water at 48- to 72-hour intervals. With the exception of coccidia, all calves were parasite-free at the time of infection. Oocysts were detected in the feces of calves 12, 14, 15, and 18 (control) prior and subsequent to infection. The estimated number of oocysts recovered at frequent intervals by flotation varied only slightly and, together with the findings at necropsy, indicated that these calves never had clinically significant coccidial infections.

Origin, Culture, Administration, and Dosages of Infective Larvae.—The strain of *T. axei* used was originally obtained from cattle in Virginia. It was established in lambs by Turner and Kates [13] and passed through several lambs by these workers prior to its transfer to calves. The original larvae, fed to the first lamb in the series, were predominantly *T. axei*, but a small percentage was *Trichostrongylus colubriformis*. At necropsy, over 99 per cent of the worms recovered from this lamb were *T. axei* (personal communication from Turner and Kates). Hence, it appeared that larval cultures from the aforementioned source were sufficiently pure for a satisfactory study of the effect of *T. axei* on calves. Table 1 shows the origin of the larvae employed in establishing experimental infections.

Infective larvae were obtained from 8- to 11-day-old charcoal-feces cultures and fed to calves from one to three days after isolation from cultures. After estimating the number of larvae in a suspension by the usual dilution method of counting, the desired number was administered by means of a long metal pipette. All larvae administered on a given day were from the same suspension. Ten calves were given infective larvae and 4 were held as controls.

Because of reports on the number of larvae producing fatal infection in lambs, the dosages first employed in this experiment were 50,000 and 100,000 larvae. Increasing dosages were then tested until a pronounced effect on the host was obtained. Since it was uncertain whether a particular number of larvae would have more effect if given in a single feeding or in multiple feedings, both types of administration were tried at various dosage levels. In some cases, after the first feeding had proved to have no appreciable effect, further feedings of larvae were given. Multiple doses were administered at approximately weekly intervals, except where otherwise indicated (table 1).

Clinical and Laboratory Examinations.—For two to four weeks before the first administration of larvae and throughout the investigation, calves were weighed at regular weekly intervals and blood samples were obtained for determination of hemoglobin levels and packed red cell volume per 100 cc. of blood (hematocrit). Twice weekly the number of eggs per gram (e.p.g.) in rectally obtained fecal samples was determined by the direct centrifuge-flotation (DCF) method using saturated

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TABLE 1—Infective Larvae Administered, Egg Counts, Worms Recovered, and Related Data Pertaining to Trichostrongylus Axei Infection in Calves

Animals		Larvae				Interval (da.) between		Maximum egg count per gram of feces	Interval between infection & necropsy (da.)	Worms recovered (Trichostrongylus spp.‖)				
Calf No.	Age when infected or re-infected (mo.)	Date(s) of administration of larvae	Total No. fed	No. per dose	No. of doses & prior passages* of strain	Initial infection & beginning of patency	Beginning of patency & maximum egg count			From abomasum (No.)	From small & large intestines (No.)	Total (No.)	In relation to total larvae administered (%)	In relation to total No. recovered (%)
10	3.0	Jan. 23	100,000	100,000	1–S^6	21	28	1,067	83	29,800	300	30,100	30.0	99.0
9	3.0 8.0	Jan. 23 June 15	500,000	50,000 450,000	1–S^6 1–S^7	25	17	516	182	19,260	600	19,860	4.0	97.0
16	3.0	May 3 to June 4	216,800	37,500 19,100 20,000 70,000	1– 1– 1– 2– } C^1	30	30	410	80	13,660	400	14,060	6.5	97.2
13	4.5	May 27	500,000	500,000	1–S^7	22	25	1,030	48	137,300	1,600	138,900	27.8	98.8
15	3.0	May 3 May 27 to July 24	500,000-600,000	100,000 100,000	1–C^1† 5–C^1†	26	33	410	129	11,200	2,800	14,000	2.0 to 2.8	80.0
12	4.0 8.0	May 19 Sept. 10 Sept. 28	679,000	350,000 104,000 225,000	1–S^7 1– } C^2‡ 1– }	24	24	1,180	180	167,200	2,100	169,300	25.0	98.8
28	3.5	June 4	750,000	750,000	1–S^{11}	21	26	907	59	116,600	3,150	119,856	15.9	97.3
19	6.0	Aug. 11	1,000,000	1,000,000	1–C^1§	28	23	1,458	56	138,400	1,210	139,610	13.9	99.1
21	4.5	July 10 to July 31	1,000,000	250,000	4–C^1§	24	29	1,080	68	110,500	1,390	111,890	11.8	98.8
31	3.5	June 4	1,500,000	1,500,000	1–S^{11}	19	28	536	49	116,800	11,700	128,500	8.6	90.9

* Animal passages: S = sheep; C = calf; exponent indicates number of passages. † Culture obtained from calf 9. ‡ Culture obtained from calf 21. § Culture obtained from calf 13. ‖ For details as to proportions of *T. axei* and *T. colubriformis*, see text; the percentages shown in the last column indicate the approximate purity of the infections for *T. axei*.

sodium chloride. All calves were observed daily for the appearance of clinical symptoms.

At the beginning of the experiment, fecal consistency (table 2) was judged visually only and the feces were classified as normal, mushy, or diarrheic. Later in the experiment, such visual classification was supplemented by determinations of the percentage of water content. Determinations were made by placing 4- to 8-Gm. samples of fresh rectally obtained feces in shallow glass dishes and desiccating them in an oven at 60 C. for forty-eight hours. This method provides only an approximation of the total water in a given sample. However, since all samples were treated alike, it was considered satisfactory for comparative purposes.

Examination at Necropsy.—Test calves which did not die were slaughtered when death appeared imminent or shortly after the maximum possible effect apparently had been produced by their infections. The method of postmortem examination, which was carried out as soon as possible after death or slaughter, was the same for all calves. The abomasum, small intestine, and large intestine were thoroughly washed in water and the entire contents and washings were preserved in formalin without screening. The abomasum and upper portion of the small intestine were examined for evidence of macroscopic lesions and tissue specimens were preserved for sectioning and microscopic study.

Numbers of worms recovered in each case were estimated from the number in a minimum of 3 aliquots of each standard-volume suspension of the abomasum and intestinal contents and washings. To estimate the proportion of each species of Trichostrongylus and possible contaminants in the various infections, the abomasum material was completely examined for large worms, like Haemonchus, and all male worms in the aliquots of the abomasum material from all calves and those of the intestinal material from calves 13, 28, and 31 were identified.

RESULTS

Parasitological Data.—Egg Counts.—Eggs were first found in the feces (table 1) as early as nineteen days (calf 31) and as late as thirty days (calf 16) after the first administration of infective larvae. No clear relationship between the length of the prepatent period (interval between infection and first appearance of eggs in feces) and the number of larvae administered was evident. The length of the patent period was not determined in any case. The longest interval between a single feeding of larvae or between a final feeding in a series and necropsy was eighty-three days (calf 10); at that time, a patent infection was still present.

The maximum egg count for calves that received single doses varied from 516 e.p.g. (calf 9, initially fed 50,000 larvae) to 1,458 e.p.g. (calf 19, fed 1,000,000 larvae). Although the maximum count (1,067 e.p.g.) for calf 10, fed 100,000 larvae, was nearly twice as great as that for calf 9, the peak count for the calves that received single doses was not proportional to the number of larvae administered. In both single and multiple infections, the peak egg count divided by the number of larvae administered gave a series of quotients which tended to decline with increased dosage. From such comparisons as can be made, namely between calves 13 and 15 and between calves 19 and 21, it appeared that where approximately a like total number of larvae were administered in a single dose versus multiple feedings, the single administration resulted in a higher egg count.

TABLE 2—Effect of Trichostrongylus Axei Infections on Fecal Consistency

Calf No.	Appearance* or water content of feces		Period (days) in which abnormal feces were passed and frequency of passage‡
	Before infection	After initial infection†	
10	Normal	Normal	.....
9	Normal	Normal	.....
11 (Control)	Normal		.....
16	78.1 (74.7 to 81.5)	79.7 (74.0 to 85.6)	10 AP to 17 AP (intermittent—5 of 7 days).
13	75.8 (71.0 to 78.8)	79.0 (75.0 to 82.2)	.....
15	77.7 (74.3 to 82.3)	79.0 (77.0 to 83.0)	.....
12	78.1 (74.0 to 79.9)	79.0 (71.0 to 83.0)	.....
18 (Control)	78.0 (75.0 to 82.1)		.....
19	79.3 (77.0 to 81.9)	91.5 (81.0 to 95.0)	8 AI to slaughter (continual).
21	74.4 (72.9 to 75.5)	85.3 (79.0 to 89.0)	19 AI to slaughter (continual).
22 (Control)	78.4 (71.0 to 82.4)		.....
28	78.5 (77.0 to 79.5)	82.4 (79.3 to 87.9)	12 AI to slaughter (intermittent—36 of 47 days).
31	76.4 (74.1 to 78.0)	94.8 (91.0 to 99.5)	1 AI to death (continual).
32 (Control)	77.3 (75.8 to 82.0)		.....

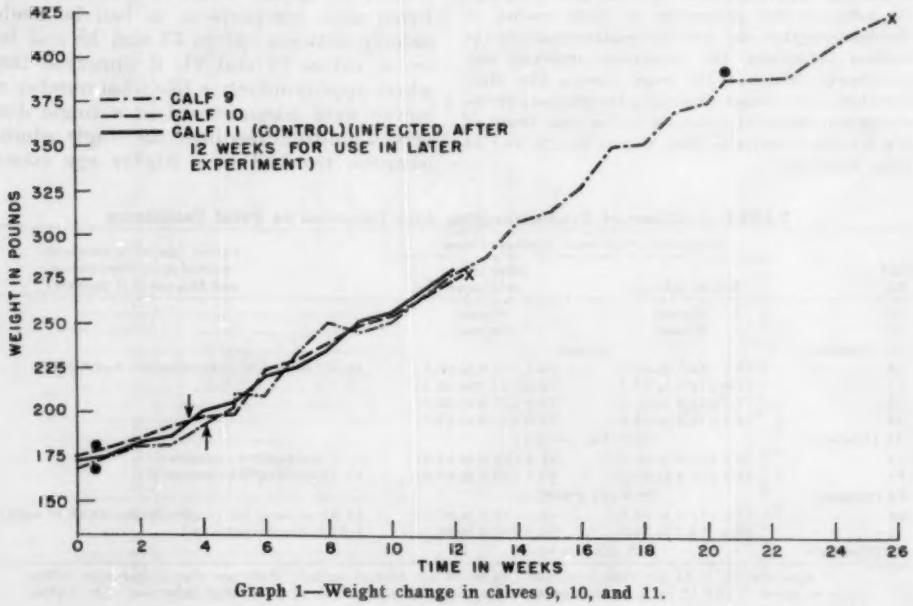
* Appearance: <84 per cent = normal; 84 to 90 per cent = mushy; >90 per cent = diarrheic. † The feces of calves 9 and 12 were normal at all times following reinfection. ‡ AI = after infection; AP = after beginning of patency.

The time of maximum egg count varied from seventeen to thirty-three days after the beginning of patency and did not appear to be related to the number of larvae initially fed. There was, however, a tendency for the peak to appear earlier when a single dose of larvae was administered.

Worms Recovered.—Since no two test calves were examined at the same interval after initial infection, only limited comparisons are possible as to the percentage of development obtained at differenct levels of exposure to infection. In calves that received a single feeding of larvae, there was evidence that the percentage of development was lower at the higher levels of dosage. The worms recovered from calf 10, eighty-three days after infection, represented 30 per cent of the 100,000 larvae administered, whereas those recovered from calves 13, 28, 19, and 31, which received progressively more larvae and were slaughtered between forty-eight and fifty-nine days after infection, represented 27.8, 15.9, 13.9, and 8.6 per cent, respectively, of the larvae given. Four of the 10 calves receiving larvae had relatively small numbers (14,000 to 30,000) of worms, but the other 6 had enough (112,000 to 170,000) to permit classifying their worm loads as "moderate to heavy" in view of reports as to the size of naturally acquired infections.

Purity of Infections.—All of the male worms found, their number varying from 36 to 523, in the aliquots from the abomasum contents from 7 of the calves were *T. axei*. Two of 62 males from calf 9, 1 of 545 from calf 13, and 1 of 458 from calf 31 were *T. colubriformis*. If the conventional assumption of a 50:50 sex ratio for both species is made, these findings indicate that 3.2, 0.2, and 0.2 per cent, respectively, of the Trichostrongylus from the stomachs of these calves were *T. colubriformis*. The presence of this species in the stomach is rather unusual and may have been due to migration after death of the host.

If the assumption were arbitrarily made that all of the Trichostrongylus recovered from the intestines (table 1) of all calves were *T. colubriformis* and the aforementioned percentages of abomasum contamination by this species in 3 of them also is taken into account, the purity of the various infections for *T. axei* would be 80 per cent in calf 15 and would range from a minimum of 90.7 per cent (calf 31) to a maximum of approximately 99 per cent (calves 10 and 19) in the remaining 9. However, examination of small numbers of

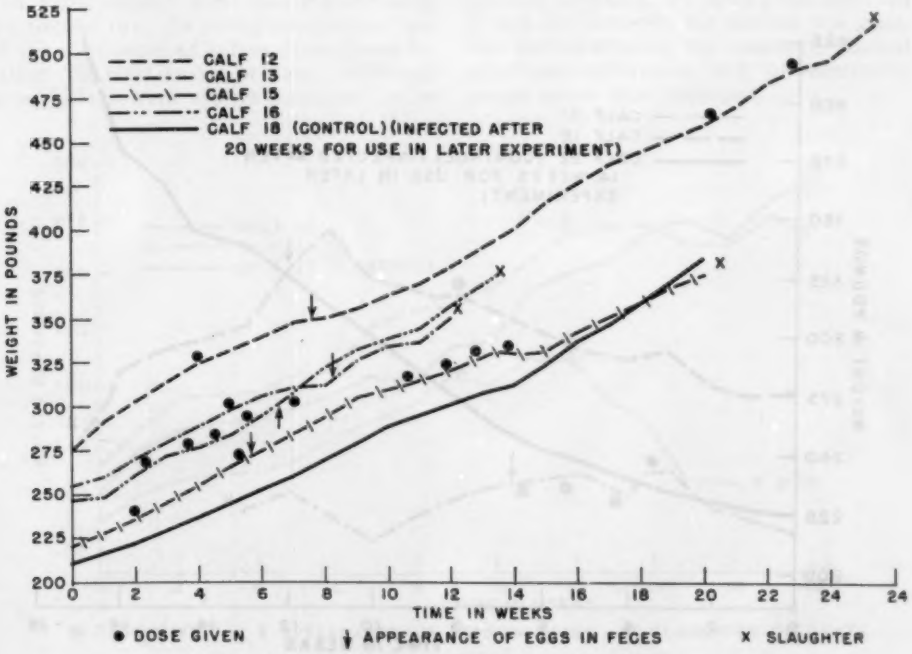


Graph 1—Weight change in calves 9, 10, and 11.

males from the small and large intestines of calves 13, 28, and 31 showed that only 30, 74, and 61 per cent of them, respectively, were actually *T. colubriformis*, thus indicating, for example, that 94.3 rather than 90.7 per cent of all Trichostrongylus recovered from calf 31 were *T. axei*. Calves 15, 16, 19, and 21 received larvae of similar origin (table 1) and the finding of proportionately more worms in the intestines of calf 15 probably does not mean that as many as 20 per cent of the worms recovered from it were *T. colubriformis*. While calf 28 harbored 106 *Haemonchus contortus*, none was recovered from any of the other test calves and no species not thus far mentioned was recorded. This relatively slight degree of contamination thus appears insufficient to have materially affected the clinical effects observed in this experiment.

Effects of Infection.—Blood Levels.—There was no significant difference between the packed red cell volume per 100 cc. of blood (hematocrit) and hemoglobin levels of infected calves and their controls. No other determinations on the blood were made.

Weight Changes, Morbidity, and Mortality.—Calves 9 and 10 (graph 1) and calves 12, 13, 15, and 16 (graph 2), which received less than 750,000 larvae (table 1), gained weight at approximately the same rate as their respective controls. During the intervals between the time of initial infection and slaughter, calves 9 and 10 gained an average of 8.6 and 9.4 lb. per week, respectively, whereas calf 11 (control) gained 10 lb. per week from the time that calves 9 and 10 were infected until the time calf 10 was slaughtered. Unfortunately, at that time it was necessary to infect calf 11 for use in later work and, consequently, calf 9 lacked a control for the last ninety-nine days before slaughter. The average weight gains for calves 12, 13, 15, and 16 were 7.9, 8.1, 7.7, and 10.2 lb. per week, respectively, whereas the control (calf 18) gained 8.6 lb. per week from the time calf 15 was initially infected until the control was also infected for use in a later experiment. The average weight change per week in calves 12 and 21 (1,000,000



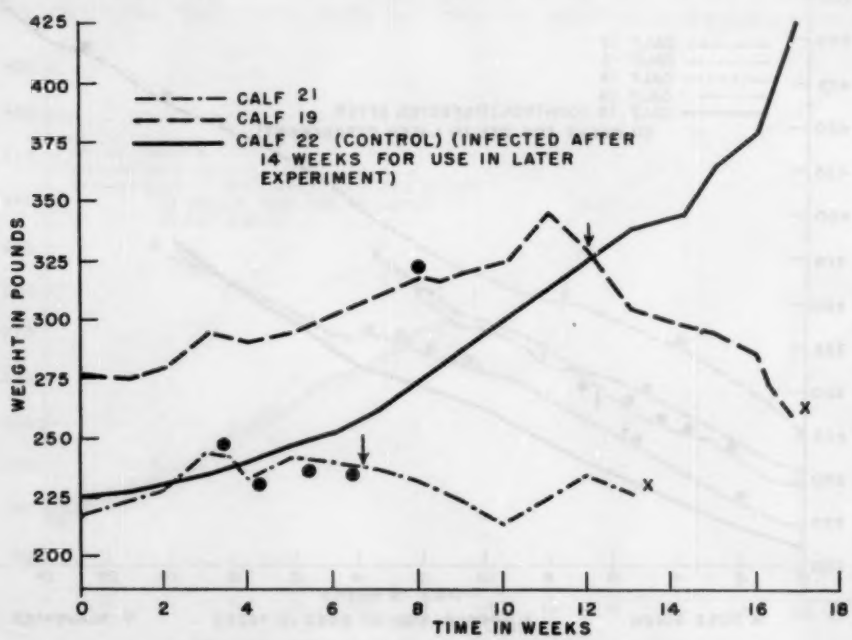
Graph 2—Weight change in calves 12, 13, 15, 16, and 18.

larvae in single and multiple doses, respectively) and calves 28 and 31 (single doses of 750,000 and 1,500,000 larvae respectively), however, was entirely different from that in calves given smaller doses.

From the time of infection to slaughter, calf 19 (graph 3) lost 57 lb. (average, 7 lb. per week) and calf 21 lost 17 lb. (average, 1.8 lb. per week), whereas the control calf gained 147 and 102 lb. (average, 18.4 and 10.5 lb. per week, respectively) during the corresponding periods. Calf 28 (graph 4) gained only 30 lb. (average, 3.5 lb. per week) and calf 31 lost 46 lb. (average, 7.4 lb. per week), whereas their control gained 72 and 57 lb. (average, 8.7 and 8 lb. per week, respectively) during corresponding intervals. The 4 calves given 750,000 or more larvae became emaciated, listless, and weak; these conditions were least pronounced in calf 28 and most pronounced in calves 19 and 31. Calf 19 was slaughtered *in extremis* and calf 31 died forty-nine days after infection.

Feed Consumption.—None of the 6 calves (No. 9, 10, 12, 13, 15, and 16) that received 678,000 larvae or less (table 1) was ever off feed or in poor condition. Six pounds of hay was rejected by calf 13 during the third week of its infection and occasionally 1 or 2 lb., mostly course stubble, was rejected by others; however, these small amounts were not considered significant. Calves 19, 21, 28, and 31, all of which received 750,000 or more larvae, were definitely off feed. Calves 31, 28, and 19 first rejected alfalfa three, seven, and sixteen days, respectively, after infection; however, calf 21 did not reject alfalfa until four days after the infection became patent. During the interval between infection and slaughter, the over-all amount rejected by calves 19, 21, and 28 was less than 14 per cent; however, calf 31 rejected 64.4 per cent of the alfalfa provided. The amount rejected by calves 28 and 21 did not increase appreciably from week to week, but the amount rejected by calves 19 and 31 gradually increased until 65.3 and 91.8 per cent, respectively, was rejected during the week preceding necropsy.

Consistency of Feces.—The water content (table 2) in the feces of calves given less than 750,000 larvae increased slightly at various intervals following administration of larvae. However, with the excep-



Graph 3—Weight change in calves 19, 21, and 22.

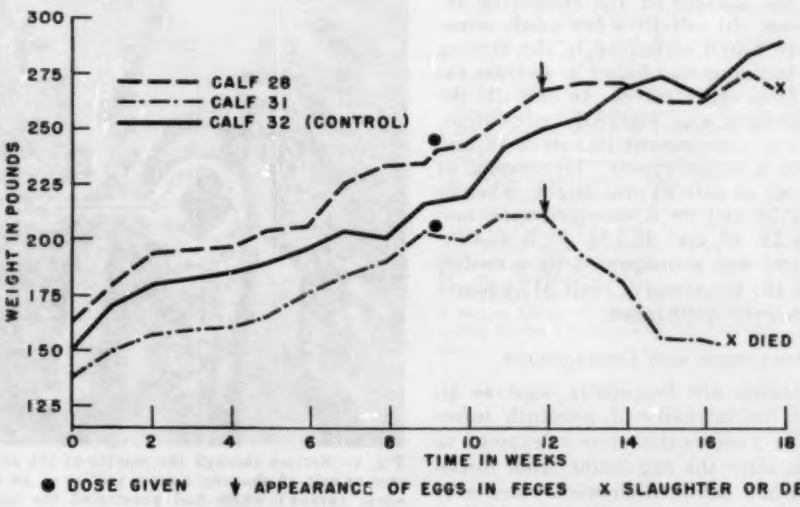
tion of calf 16, the feces were never fluid enough to be designated as mushy. The feces of this calf were intermittently mushy between the tenth and seventeenth days after the beginning of patency. Eight days after administration of larvae, the feces of calf 19 became mushy and continued so for seventeen days. Its feces were then diarrheic until slaughter. At one time, forty-six days after infection, the water content reached 95 per cent. The feces of calf 31 were diarrheic from the first day after infection until death, at which time the water content was 99.5 per cent. The feces of calves 28 and 31, although either intermittently or continually mushy, respectively, never became diarrheic.

Gross Pathology.—Gross pathological changes were observed in the abomasums of all infected calves, except calf 9. This calf, however, was slaughtered nearly six months after initial infection and apparently had time to recover from the effects of infection.

Lesions about 1 or 2 cm. in diameter, similar to the "ringworm-like" lesions observed in the stomachs of horses by Roth and Christensen,[9] were found irregularly distributed over the entire mucosal surface of the abomasum of calves given doses totaling 750,000 larvae or less. Although these lesions were always estimated to be less than 150 in number, they were most prevalent in calves 13, 21, and 28; less prevalent in calves 15 and 16; and least prevalent in calf 19. Hence, their frequency tended to be roughly correlated with the number of larvae administered and to the elapsed time between infection and slaughter. The lesions in the abomasum of calf 21, however, were nearly twice the size of those present in the abomasums of calves 19 and 31.

The mucosa of the abomasums of calves given 750,000 larvae or more appeared greatly inflamed; however, the intensity of the inflammation was most pronounced in calves 19 and 31. The reddish purple color was usually uniform over the entire surface of the abomasum, but in calf 21 it was mostly confined to the folds of the abomasum and diffuse petechial hemorrhages were visible over the relatively normal areas of the mucosa. The first foot of the small intestine of calf 21 also showed a slight inflammation.

In calves 19 and 21, the entire surface of the abomasum was covered with a layer of thick mucus. The mucosa beneath it was roughly granular and somewhat corrugated or wrinkled. No mucus was observed in calf 31; however, the mucosa was granular and corrugated, the mesentery showed gelatinous infiltration, and the mesenteric lymph nodes were enlarged.



Graph 4—Weight change in calves 28, 31, and 32.

Histopathology.—No changes were found in the small intestine. Sections from the abosamums of all infected calves, except calf 9, showed sloughing of the superficial epithelium (fig. 2) which was most pronounced and extensive in calves 12, 19, and 31. In sections showing desquamation, masses of cellular debris were frequently seen on the surface of the remaining intact mucosa. In calf 19, a few adult nematodes were found embedded in the mucosa (fig. 2), but none was found in sections examined from other calves. In calf 21, the tunica propria was markedly edematous. Edema was also present in calves 13 and 16 but to a lesser extent. Hyperemia of the mucosa in calf 21 was slight, whereas in calves 15 and 28 it was moderate, and in calves 12, 19, and 31 (fig. 1) it was extensive and was accompanied by a moderate (calf 12) to extensive (calf 31) amount of lymphocytic infiltration.

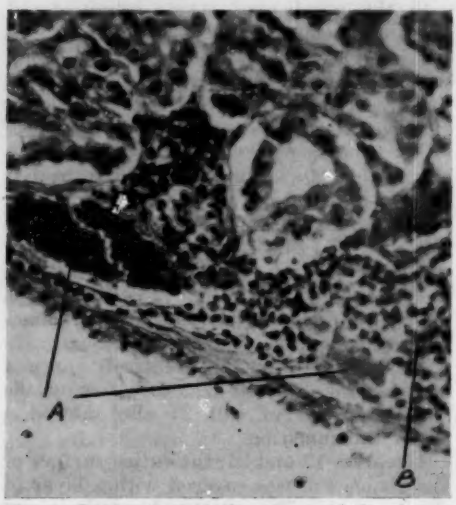


Fig. 1—Section through the mucosa of the abomasum of calf 19 showing (a) hyperemia and (b) lymphocytic infiltration.

DISCUSSION AND CONCLUSIONS

Egg counts are frequently used as an index of the intensity of helminth infections. The 2 calves that were re-exposed to infection after the egg count from initial infection had subsided showed evidence of having developed resistance to reinfection. Calf 9 was passing 220 e.p.g. at the time it was reinfected. During the thirty-nine days between re-exposure and slaughter, the count never rose above 220 e.p.g. and had decreased to 70 e.p.g. at the time of slaughter. When calf 12 was re-exposed to infection the egg count was 566 e.p.g. The counts for this calf likewise never rose above the level at the time of re-exposure and had decreased to 104 e.p.g. at the time of necropsy forty-seven days later. As shown in table 1, the maximum egg counts following initial infection with single doses were reached between forty and fifty days after infection. If calves 9 and 12 had not been resistant, it would be expected that the egg counts would have risen to levels higher than those at the time of re-exposure.

Retarded growth rate, weakness, lack of appetite, and watery feces were evident when calves were originally infected by

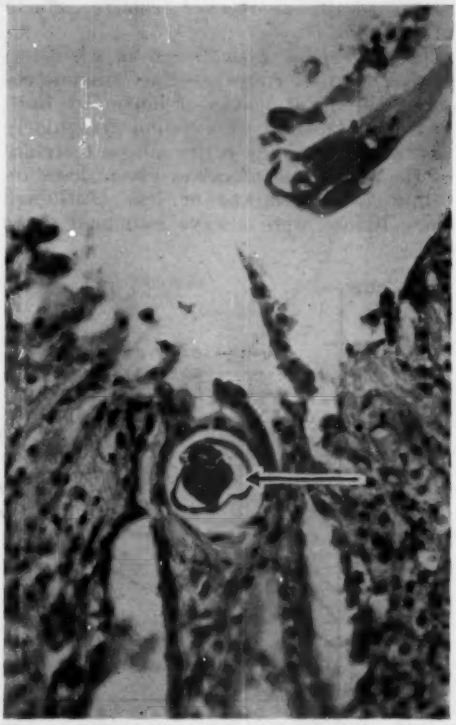


Fig. 2—Section through the mucosa of the abomasum of calf 19 showing a cross section of an adult worm (arrow) which had penetrated the mucosa with sloughing of the adjacent tissue.

single or multiple doses of larvae between 750,000 and 1,500,000 in number. Under the conditions of this experiment, it appeared that 750,000 larvae were necessary to produce clinical symptoms in 3- to 6-month old calves. Since calf 31 died forty-nine days after infection and calf 19 was *in extremis* after fifty-nine days, it is quite probable that the minimum dose necessary to produce death is close to 1,000,000 larvae. Doses of less than 750,000 larvae did, however, produce pathological changes in the abomasum; the intensity varying roughly with the number of larvae administered. Calves 12 and 13, although they received smaller larval dosages and showed no clinical effects, had worm loads comparable to the 4 calves that showed symptoms. From the information available in the present report and that of Turner and Kates,[13] it appears that it is necessary to administer much larger numbers of *T. axei* larvae to calves than to lambs in order to produce comparable clinical effects. Turner and Kates produced a fatal case of *T. axei* infection in lambs by administering 50,000 larvae. Gibson[7] reported weight loss followed by death of 3 of 6 lambs fed 100,000 larvae in daily doses of 4,000 each, when the animals were on a low plane of nutrition. It is also of interest that Andrews,[1] who worked mainly with *T. colubriformis* found minimum doses of 78,000 and 115,000 larvae fatal to a kid and a lamb, respectively.

SUMMARY

When 10 Holstein-Friesian calves, 3 to 6 months old, were each given from 50,000 to 1,500,000 Trichostrongylus larvae (95% estimated to be *Trichostrongylus axei*), the results were as follows:

1) The prepatent period for *T. axei* in single and multiple infections was nineteen to thirty days. The maximum egg count was usually observed twenty-six to thirty days after patency and in one instance as early as seventeen days.

2) Resistance to heavy reinfective doses, four to five months after their egg counts from initial infection had subsided, was shown by 2 calves.

3) Loss of weight, weakness, loss of appetite, and watery feces were evident when calves were initially given single or multiple doses of 750,000 or more larvae. A calf given 1,000,000 larvae was *in extremis* at fifty-nine days and 1 that received 1,500,000 larvae died forty-nine days after infection. Although not producing clinical symptoms, exposure to less than 750,000 larvae produced gross pathological changes in the abomasum, namely "ringworm-like" lesions, inflammation, and corrugation of the musoca of the abomasum; also histopathological changes, such as sloughing of the epithelium, hyperemia, and lymphocytic infiltration. These changes varied roughly with the number of larvae administered.

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A Sampling Tube for Rumen Fluid

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THE SAMPLING tube (fig. 1) has solved the problem of obtaining portions of rumen fluid from different areas of the paunch without admixture with material from adjacent areas during the act of sampling.

The tube (aluminum) with the larger diameter is placed over the tube (stainless steel) with the smaller diameter and rotated until the slots near the lower end of the tubes are opposite to each other, thus closing the opening. The tubes then are inserted into the paunch through a fistula and directed to the area from which a sample is to be removed. Once there, the tubes are rotated until the slots coincide, allowing rumen fluid to enter the inner tube. The tubes again are rotated to close the opening, are removed from the paunch, and are inverted to allow the liquid to be poured through the upper opening into the container used to hold the sample.

The diameter of either tube can be any reasonable figure, provided its mate is a loose but watertight fit. Both tubes have aluminum plugs in the lower ends and are open at the top. The length of the tubes is not critical nor is the position of the slots. The outer tube in the illustration is about 2½ feet long and about 1½ inches in outside diameter. The inner tube is 6 inches longer. Although not necessary, file marks at the top of the shorter tube and on the exposed side of the longer tube assist in indicating slot positions.

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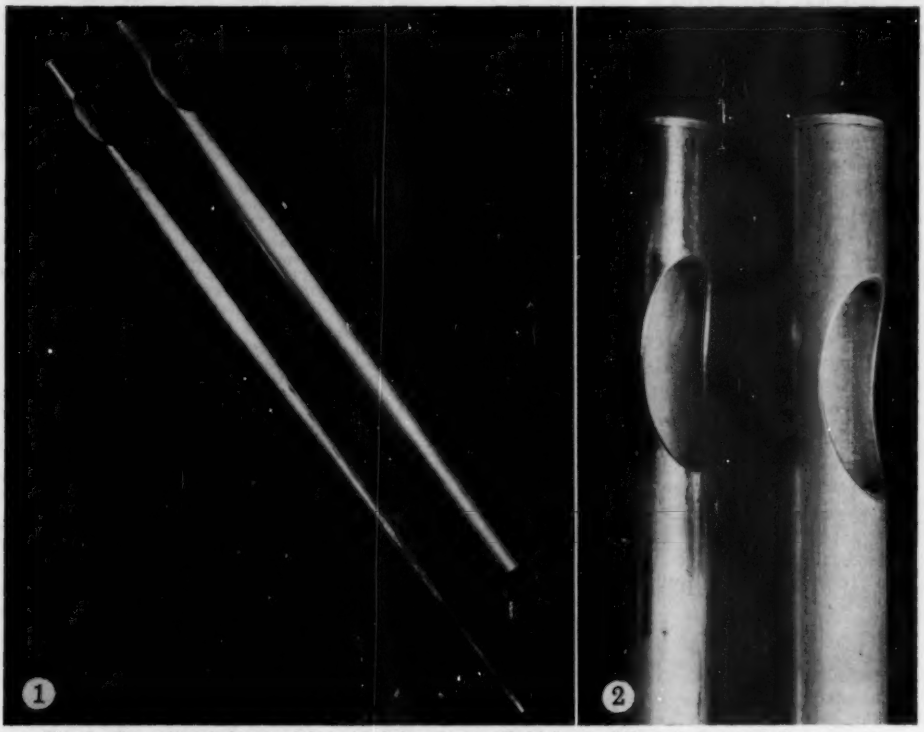

Fig. 1—Sampling tube used for obtaining rumen fluid from different areas of the paunch without admixture of material from adjacent areas.
Fig. 2—Closeup of slots of the outer (right) and inner tubes.

Physiological Studies of the Vagal Nerve Supply to the Bovine Stomach. I. Comparison of Responses in Milk-Fed and Roughage-Fed Calves, Using a Chronic Intrathoracic Vagal Electrode Technique

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ACUTE BLOAT, a common digestive disorder of ruminants sometimes occurring after the ingestion of legume plants, has in recent years drawn the attention of an increasing number of people who raise and care for livestock. The variety of theories in regard to the cause of the condition, and the treatments recommended, indicate a lack of basic knowledge about the condition.[1,2] The experiments here recorded were conducted in an attempt to find new methods of study for and to add to the basic knowledge of the physiology of the bovine stomach.

METHODS

Seventeen dairy-type male calves were used for the experiment. Fourteen of the calves were started on the experiment at birth, the remaining 3 at 2 to 3 months of age. Six of the calves were fed an ordinary ration consisting of hay, ground corn and oats, and whole milk. Nine of the calves were kept on wire screen and were fed only whole milk with a vitamin and mineral supplement. Two calves were started on the milk-only ration, then changed over to the milk, hay, and grain ration along with rumen inoculations from adult cows via stomach tube, during the course of the studies. This difference in feeding regime was adopted in view of the work of others showing marked differences in rumen development when so fed.[3-7]

Thoracotomy was performed on all calves for the purpose of applying vagal electrodes, as described below. Following recovery from surgery, spontaneous and electrically stimulated gastric motility patterns were studied in the unanesthetized state by simultaneous fluoroscopy and intraruminal pressure recording via a modified Brody-Quigley tube.[8-10] A comparison was made between the response to vagal nerve stimulation of the rumens of the milk-fed and of the roughage-fed calves. Pressure changes in the anterior ventral sac of the rumen were measured and recorded by means of an unbonded strain-gauge transducer * and direct-writing recorder.** The electrodes were made of pure silver wire (18 gauge) soldered to plastic-coated copper wire (fig. 1).

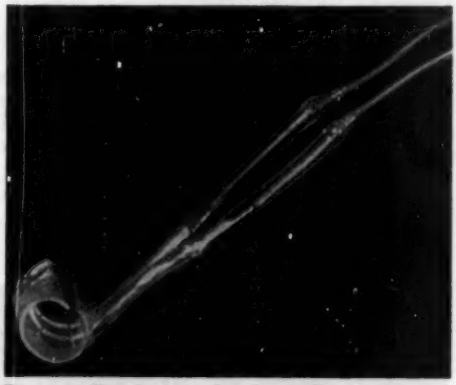

Fig. 1 — Vagal electrode. Pure silver wires and large-bore polyethylene shield at lower left. Plastic-coated copper wire at upper right. Transparent insulation material at exposed portions of wires is colorless fingernail polish.

For surgery, the calves were anesthetized with intravenous pentobarbital sodium. The incision (18–20 cm. in length) was made between ribs 7 and 8 on the left side. The diaphragmatic lobe of the left lung was reflected anteriorly and the left mediastinal pleura was broken to apply the electrodes on the dorsal and ventral vagal nerve trunks.[11-13] The point of application was 3–6 cm. anterior to the diaphragm (fig. 2). Artificial respiration was provided by use of a continuous intratracheal flow of pure oxygen from a large tank, using a polyetheylene catheter.† The free ends of the electrode wires were brought out of the thorax between ribs 7 and 8 and anchored in the subcutis.

One month after thoracotomy, motility studies including nerve stimulations were tried, using an

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* Model P-23-A, The Statham Laboratories, Inc., Hato Rey, Puerto Rico.

** Model 60, The Sanborn Co., Cambridge, Mass.

† P E 280, Clay-Adams Co., Inc., New York, N. Y.

electronic square wave stimulator.‡ The recording tube inside a radiopaque horse catheter was passed through the nose into the ventral sac of the rumen. The position of the recording tube was determined by fluoroscopy. Motility studies were made on trained calves at approximately one-month intervals. An average of three trials was made on each calf. The calves were trained by repeatedly leading them with halters, placing them in the experimental stocks for several hours at a time after passing nasal catheters to the stomach. Postmortem examinations were later performed on each calf and sections for histological study were made of the vagal nerve trunks anterior to, inside of, and posterior to, the point of application of the electrodes, using Holmes' silver technique[13] and Crossmon's trichrome technique.[14]

Results

Eleven of the 17 calves made complete recoveries from the thoracotomy and were used for stimulation trials. These were divided as follows: on milk only from birth—5 calves; on roughage plus milk from birth—4 calves; and on milk from birth to 60 days of age, then on roughage—2 calves. The fates of the remaining 6 calves, not used for experimental trials, were as follows: death during or shortly following surgery—3 calves; pneumonia—2 calves; and destroyed because of chronic arthritis—1 calf.

Simultaneous fluoroscopic and manometric observations indicated that spontaneous rumen motility of the roughage-fed calf, in general, was more regular and contractions were stronger than in the rumen of the milk-fed calf. Recorded amplitude of contractions increased as roughage-fed calves grew older; rumen motility in the milk-fed calf showed little change with age over the period studied (birth to 90 days of age). At times, milk-fed calves appeared to show no spontaneous motility of the rumen.

Electrical stimulation, using 10 to 35 volts, with a pulse frequency of 20 cycles per second, 0.5 millisecond duration of pulse, produced characteristic changes in the motility of the rumen of both milk-fed

‡ Model 751, American Electronics Laboratories, Inc., Philadelphia, Pa.

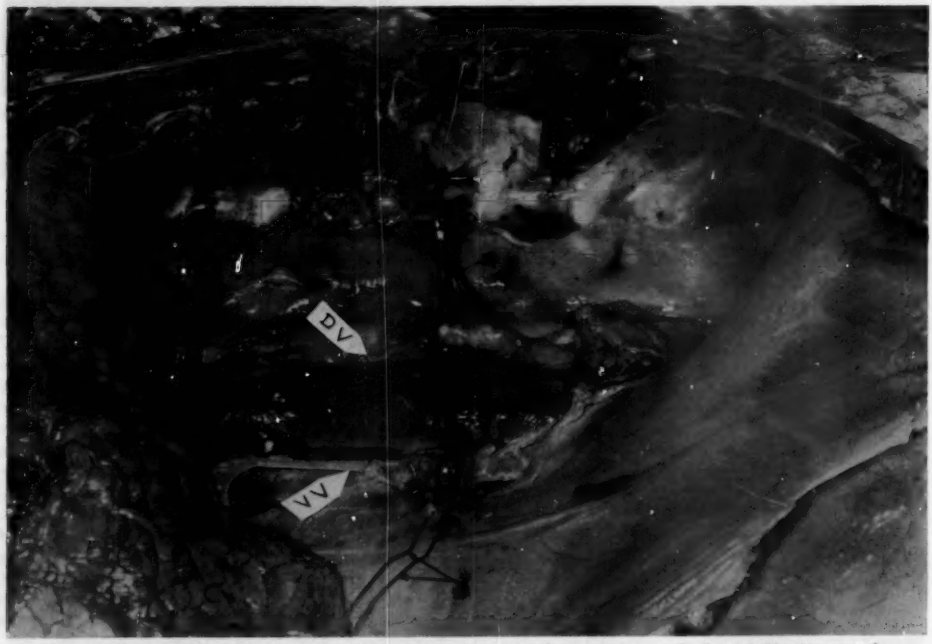


Fig. 2—Lateral view of electrode placement areas exposed at necropsy. Reflected diaphragmatic lobe of left lung at left. Sectioned diaphragm at right. Esophagus at center between dorsal vagal trunk (DV) and ventral vagal trunk (VV). Dorsal (a) and ventral (b) vagal electrodes at center.

and roughage-fed calves. In general, stimulation increased the rate of contraction of the ventral rumen sac in both categories of calves. In some cases, the amplitude of contraction was increased by stimulation; in other cases, it was decreased. Duration of the contraction of the ventral sac during stimulation varied. Stimulation with higher voltages (40–60 volts) was accompanied by evidence of reticular and ruminal spasms.

Milk-fed calves showed contractions of the ventral sac having amplitudes up to 5 mm. of mercury pressure; roughage-fed calves, up to 25 mm. of mercury pressure. Changing the diet of 2 calves from milk to roughage gradually changed spontaneous and stimulated motility patterns from those characteristic of the milk-fed group to those characteristic of the roughage-fed group. The lag for full development of roughage-fed motility appeared to be approximately sixty days (table 1).

Calves in which electrode wires were intact (16 to 101 days after operation) showed, upon stimulation, no external signs of feeling or being distressed by the stimuli used. The breaking of an electrode, on the other hand, was evidenced by skeletal muscular twitching, particularly of the left thoracic region, when the stimulator was turned on. Signs of apprehension (switch-

TABLE 1—Summary of Trials of Spontaneous and Stimulated Motility Patterns of the Ventral Rumen Sac of Milk-Fed and Roughage-Fed Calves

					Type of Contraction *								
					I**		II		III		IV		
Calf (No.)	Age (days)	Diet	No. of trials	Type trial	Dur., sec.	Rate/ min.	Dur., sec.	Rate/ min.	Dur., sec.	Rate/ min.	Dur., sec.	Rate/ min.	Remarks
1007	153–178	Roughage	3	Spon.	....	....	5.1	0.8	11.0	1.0	....	....	
				Stim.	....	....	5.4	1.3	9.5	1.7	....	....	Increased rate
1061	115–123	Roughage	2	Spon.	5.0	0.6	7.5	0.5	8.0	0.6	....	....	
				Stim.	4.5	3.1	3.6	2.0	6.0	0.5	....	....	Increased rate
1088	62–83	Roughage	2	Spon.	6.0	0.3	8.0	0.2	10.0	0.6	....	....	
				Stim.	4.0	1.4	5.5	1.6	8.5	1.0	8.5	0.9	Increased rate and amplitude
1093	50–78	Roughage	3	Spon.	5.5	1.1	7.0	0.5	7.0	0.6	....	....	
				Stim.	4.3	1.2	5.0	2.5	8.0	0.7	....	....	Increased rate
1062	30	Milk	1	Spon	5.3	1.5	....	....	....	....	....	....	
				Stim.	2.4	2.8	....	....	....	....	....	....	Increased rate, irregular pattern
1102	45–69	Milk	2	Spon.	4.0	0.8	....	....	....	....	....	....	
				Stim.	5.0	3.8	....	....	....	....	....	....	Increased rate, irregular pattern
1103	44	Milk	1	Spon.	5.0	1.4	....	....	....	....	....	....	
				Stim.	8.0	3.0	....	....	....	....	....	....	Increased rate, irregular pattern
1104	51	Milk	1	Spon.	3.0	1.8	....	....	....	....	....	....	
				Stim.	7.0	4.0	....	....	....	....	....	....	Increased rate, irregular pattern
1105	45	Milk	1	Spon.	2.5	2.4	....	....	....	....	....	....	
				Stim.	3.0	4.0	....	....	....	....	....	....	Increased rate, irregular pattern
1091	57	Milk	1	Spon.	6.0	1.1	....	....	....	....	....	....	
				Stim.	4.0	3.0	7.0	1.0	....	....	....	....	Increased rate and amplitude, irregular pattern
	58–76	Roughage	1	Spon.	3.5	1.5	....	....	....	....	....	....	
				Stim.	4.0	4.0	6.0	2.0	....	....	....	....	Increased rate and amplitude
	77–121	Roughage	1	Spon.	5.0	0.3	10.0	0.3	10.0	0.8	....	....	Spontaneous motility increased
				Stim.	Wires broken.								
1092	57	Milk	1	Spon.	5.0	0.8	....	....	....	....	....	....	
				Stim.	6.0	3.0	....	....	....	....	....	....	Increased rate, irregular pattern
	58–76	Roughage	1	Spon.	7.0	3.0	....	....	....	....	....	....	Spontaneous motility increased
				Stim.	7.0	4.0	....	....	....	....	....	....	
	77–121	Roughage	1	Spon.	6.0	0.5	7.0	1.0	10.0	0.1	....	....	Spontaneous motility increased
				Stim.	6.0	2.0	7.5	1.0	7.5	1.0	....	....	Increased rate and amplitude

*Averages only. Records of stimulation of dorsal vagal trunk, ventral vagal trunk, and both trunks simultaneously included in average figures.

** Type I—1–5 mm. of mercury pressure; II—6–10 mm.; III—11–20 mm.; IV—21–30 mm.

ing tail, kicking at sides, swinging head) were also seen at this time. Calves were not used for the present studies unless free of these signs. The position and condition of the electrodes was in all cases investigated at necropsy.

Results of trials uncomplicated by apprehension, fatigue, or electrode breakage are summarized in table 1. In the interest of clarity, only averages of rates and durations of the various wave types in the individual animals are included in table 1; also, records of stimulation of dorsal vagal trunk, ventral vagal trunk, and both trunks simultaneously, are included in the average figures, since obvious differences in the results of these procedures were not observed.

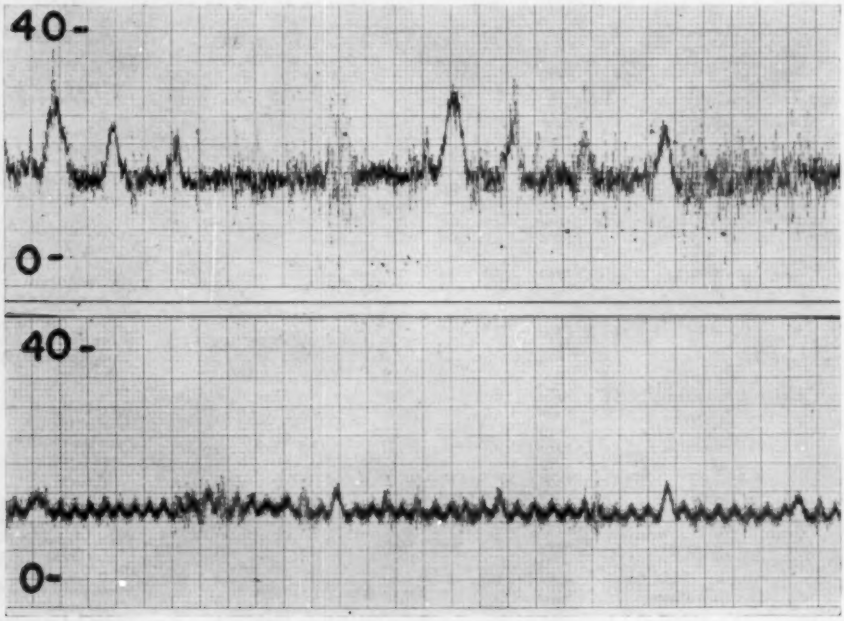
The recorded pressure waves were classified arbitrarily as follows:

Type I : 1– 5 mm. of mercury pressure
Type II : 6–10 mm. of mercury pressure
Type III: 11–20 mm. of mercury pressure
Type IV : 21–30 mm. of mercury pressure

Each period of "stimulated motility" in the table has a corresponding period of "spontaneous motility" recorded immediately preceding the "stimulated motility" period in each trial. Periods of stimulation varied from five to ten minutes.

Graphs 1 to 3 are photographs of records selected from calves on the several experimental diets. These records were considered characteristic for each group. Each record represents a five-minute period; each large division on the horizontal axis corresponds to ten seconds. The lines marked 0 and 40 indicate the calibration of the instrument at 40 mm. of mercury pressure. The calibration is such that each large division on the vertical axis is equivalent to 5 mm. of mercury pressure. The base line pressure of each record of ventral sac motility represents the approximate depth of rumen fluid above the tip of the recording tube. Both dorsal and ventral vagal trunks were stimulated simultaneously in these records.

Graph 1 shows a comparison of spontaneous ventral sac motility patterns between a milk-fed calf (1091) and a roughage-fed calf (1093). Calf 1093 was 50 days of age



Graph 1—Comparison of spontaneous ventral sac motility patterns. Calf 1093 (top) roughage-fed, 50 days of age; calf 1091 (bottom) milk-fed, 57 days of age. Note greater amplitude of rumen contractions in roughage-fed calf.
Horizontal axis, 1 large division = 10 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.

and calf 1091 was 57 days of age at this time. The smallest waves on the record of calf 1091 (approximately 0.5 second duration) were due to heart beats, as determined by auscultation. The next larger waves (approximately 4 seconds duration) were due to respiratory movements, as determined from inspection of simultaneous pneumograph records (not shown). The largest waves (6–8 seconds duration) were due to ventral sac contractions and confirmed fluoroscopically.

Graph 2 shows a comparison between spontaneous and stimulated ventral sac motility patterns in roughage-fed calf 1088 at 62 days of age. A similar comparison, in milk-fed calf 1091 at 57 days of age, is shown in graph 3.

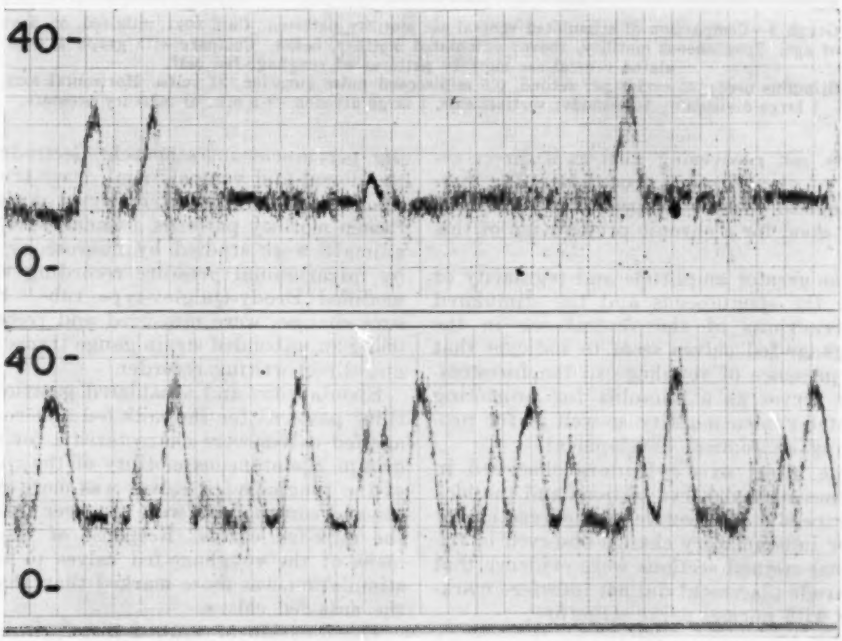
The number of times each calf could be used for stimulation studies was limited by breakdown of the electrodes. The causes for breakdown of the wires appeared to be: movement of the scapula over the dorsal vagal electrode wires as they protruded from the thorax, bending of the wires during respiratory movements, stretching forces on the wires with thoracic growth, and corrosion of the copper wires, especially near the soldered junctions.

Histologically, tissue reaction to electrode placement appeared mild. Inflammatory cells were numerous at the point of electrode application in only 1 calf (1062). In all of the other calves, normal-appearing, loose, areolar connective tissue surrounded the nerve trunks.

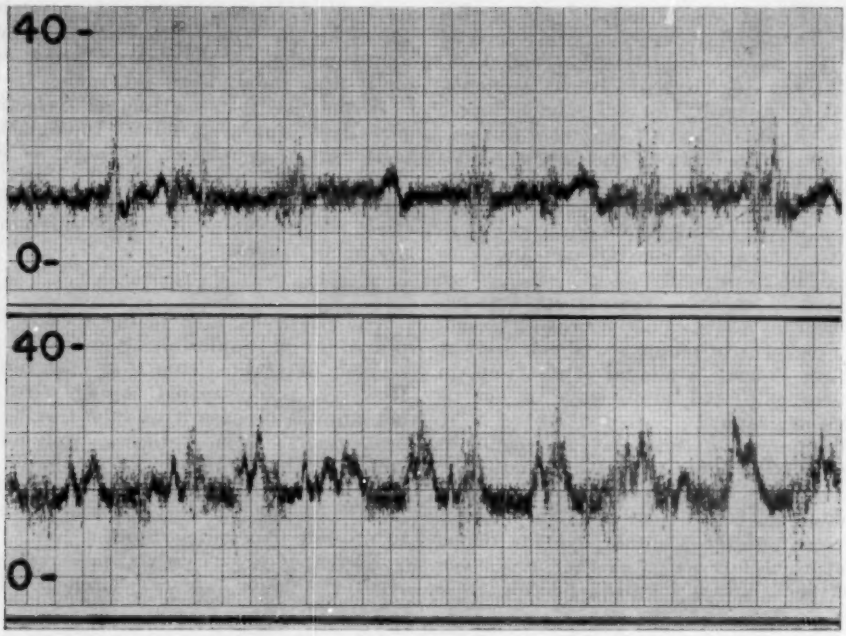
DISCUSSION

The electrodes used in these studies appeared to be nonirritating to tissue and seemed desirable from that standpoint. However, plastic-coated copper wire was not suitable because it was too fragile and broke easily. In later studies, applying the technique to the study of eructation in adult cattle, continuous silver wire was used.[15] Melted polyethylene plastic served to insulate exposed portions.

Eleven of the 17 calves made complete recoveries from surgery. Three of the 6



Graph 2—Comparison of stimulated ventral sac motility patterns. Calf 1088, roughage-fed, 62 days of age. Spontaneous motility, above; stimulated motility, below. Compare with graph 3, stimulated ventral sac motility patterns of milk-fed calf.
Stimulus used: 20 cycles per second, 0.5 millisecond pulse duration, 35 volts. Horizontal axis, 1 large division = 10 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.



Graph 3—Comparison of stimulated ventral sac motility patterns. Calf 1091, milk-fed, 57 days of age. Spontaneous motility, above; stimulated motility, below. Compare with graph 2, stimulated ventral sac motility patterns of roughage-fed calf.
Stimulus used: 20 cycles per second, 0.5 millisecond pulse duration, 35 volts. Horizontal axis, 1 large division = 10 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.

calves not recovering died as a direct result of the surgical procedure. The percentage of recovery appeared to be reasonably good for a chronic preparation of this sort.

The greater amplitude and regularity of both the spontaneous and the stimulated contractions of the ventral sac in the roughage-fed calves seem to indicate that the presence of roughage in the forestomachs serves as a stimulus for promoting greater rumen motility as well as for promoting anatomical development.

The intact axis cylinders observed in Holmes-stained nerve sections and the lack of excessive connective tissue reaction or other inflammatory change observed in trichrome-stained sections were evidence that electrode placement did not interfere markedly with normal nerve structure.

SUMMARY

Thoracotomies were performed on 17 dairy-type calves for the purpose of applying permanent intrathoracic electrodes to the dorsal and ventral vagal nerve trunks. Spontaneous and electrically stimulated rumen motility patterns, in unanesthetized animals, were studied by fluoroscopy and by intraruminal pressure recording via a modified Brody-Quigley-type tube. Pressure changes were measured and recorded using an unbonded strain-gauge transducer and direct-writing recorder.

Spontaneous and stimulated gastric motility patterns for the milk-fed and roughage-fed calves were characteristic for each group. Spontaneous motility of the rumen of the roughage-fed calves was more regular and contractions were stronger than in the milk-fed calves. Response of the rumens of the roughage-fed calves to vagal stimulation was more marked than that of the milk-fed calves.

Examination of stained histological sections of vagal nerve trunks of the experimental calves indicated that the electrodes were not markedly irritating to tissue and

that electrode placement did not alter normal nerve structure.

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Blood Phosphorus, Calcium, and Vitamin A in Range Sheep

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In connection with a project of the Department of Animal Industry and Range Management of the Montana Agricultural Experiment Station on the protein requirement of ewes wintered under range conditions, the Montana Veterinary Research Laboratory has had the opportunity to obtain some information on the phosphorus, calcium, and vitamin-A levels in the blood plasma of range ewes.

Description of the Range

The following description of the range upon which the ewes involved in this experiment were wintered is supplied through the kindness of Mr. G. F. Payne of the Department of Animal Industry and Range Management.

This winter range, known as the Chadbourne Sheep Range, is located in south-central Montana in the eastern foothills of the Rocky Mountains. It is typical of the drier foothills of southwestern and south-central Montana. It is an area of light snows and considerable wind. As a result, the vegetation is seldom covered with snow. The combination of wind and below-freezing temperatures, however, sometimes has a strong influence on the grazing habits of the animals.

Bluebunch wheatgrass (*Agropyron spicatum* (Pursh) Scribn. and Smith), Junegrass (*Koeleria cristata* (L.) Pers), sandberg bluegrass (*Poa secunda* Presl.), needle-and-thread (*Stipa comata*), and western wheatgrass (*Agropyron smithii*) are most common. There are a number of minor species, among which fringed sagewort (*Artemisia frigida* Willd.) is important because it provides some green forage throughout the winter.

An index of the use-value of the various plant species has been developed, which indicates the plants providing the major portion of the feed of the sheep. This is indicated in table 1.

TABLE 1—Index Figures Showing the Relative Volumes (in per cent) of Plants Consumed by Sheep*

Species	1950–1951	1951–1952	1952–1953
Agropyron spicatum	100	69	43
Koeleria cristata	85	100	100
Stipa comata	35	25	18
Agropyron smithii	25	19	0
Poa secunda	15	49	64
Artemisia frigida	**	**	7

* (per cent frequency in sample) (volume) — (Canadian density index) (per cent utilization) = base consumption index.[11]

Figures in this table show base consumption indexes converted to relative consumption values on a percentage basis. The heaviest used species are arbitrarily given a rating of 100 per cent.

** Utilization on *Artemisia frigida* was not determined in these years.

The carotene content of the plants was determined twice in the winter of 1952–1953, and the phosphorus content was determined three times that winter and four times the following winter, 1953–1954. The values obtained from these determinations are presented in table 2.

Experimental Procedure

In this experiment, 800 to 1,100 range ewes were used each winter for five years. They were grazed on the range as one band throughout the year. During the period between breeding and lambing, they were cut into several lots each morning for feeding of supplementary concentrates in pellet form. The lots from which blood samples were taken were designated as 0, 1, 2, 4, 5, and 6. Lot 0 received no supplement. Lots 1, 2, 4, and 6 received varying amounts of protein in pellet form, but the phosphorus, calcium, and vitamin A were approximately equalized in the various concentrates. Lot 5 was not wintered on the range but was fed mixed hay and alfalfa and a protein

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TABLE 2—Carotene and Phosphorus Content of Forage Samples from the Chadbourne Sheep Range

Species	Carotene (μg./Gm.)		Phosphorus (%)						
	Dec. 1952	Feb. 1953	Dec. 1952	Jan. 1953	Feb. 1953	Dec. 1953	Jan. 1954	Feb. 1954	Mar. 1954
Agropyron spicatum	1	0	0.05	0.05	0.04	0.04	0.05	0.03	0.06
Stipa comata	2	1	0.06	0.05	0.04	0.06	0.06	0.08	0.05
Koeleria cristata	2	3	0.06	0.06	0.05	0.10	0.12	0.04	0.05
Poa secunda	....	....	.....	.....	.....	0.09	0.18	0.06	0.06
Artemisia frigida	10	24	0.13	0.14	0.15	0.11	0.13	0.09	0.04
Chrysothamnus sp.	....	....	0.08	0.11	0.13	.....	.....	.....	.....

TABLE 3—Means of Plasma Phosphorus, Calcium, and Vitamin A for All Sheep Sampled on Each Date

Date	No. of samples	Phosphorus (mg./100 ml.)		Calcium (mg./100 ml.)		Vitamin A (μg./100 ml.)	
		Mean	Range	Mean	Range	Mean	Range
4–1–50	30	4.6	3.3–6.2	9.5	7.7–10.4	21	9–31
12–29–50	36	4.2	3.1–5.9	10.6	8.1–12.9	28	17–37
3–26–51	34	4.8	4.0–6.3	8.9	7.3– 9.8	22	12–36
1–4–52	49	4.2	2.8–5.3	8.8	7.2–10.2	31	16–39
3–24–52	49	3.8	2.9–5.0	9.1	7.6–10.6	28	20–39
11–22–52	50	3.4	2.2–5.1	8.3	6.8– 9.2	33	25–49
12–12–52	29	3.8	2.8–4.8	8.3	6.8–10.0	34	24–46
3–17–53	50	4.1	3.0–7.2	8.5	7.0– 9.6	24	12–48
7–2–53	46	5.4	3.5–6.9	8.9	7.5–10.3	26	19–39
10–9–53	41	4.0	2.7–5.4	10.3	8.7–11.8	38	27–49
12–11–53	51	4.6	3.2–5.5	10.6	8.9–12.4	32	22–46
3–29–54	52	4.4	2.7–6.5	9.0	7.8–10.3	27	17–53
Total	517	4.3	2.2–7.2	9.2	6.8–12.9	29	12–53
Standard deviations			0.9		1.0		7.3

concentrate. Blood samples were taken from sample groups of ewes in each lot at the beginning and end of the winter feeding period, late in December and late in March. The lots sampled were 0, 1, and 4 in the spring of 1950; 0, 1, 4, and 5 in 1950–1951; 0, 1, 4, 5, and 6 in 1951–1952; 0, 1, 2, 5, and 6 in 1952–53; and 0, 1, 2, 5, and 6 in 1953–1954. The blood analyses were started in the spring of 1950 and were continued through the winters of 1950–1951, 1951–1952, 1952–1953, and 1953–1954. In 1953, blood samples were taken also on July 2, just before going to the summer range, and on October 9, when the sheep were on fall range.

The number of sheep sampled in each lot was usually 10, but in some instances it was 9 or 11. The total number of ewes sampled at each bleeding was 30 in 1949–1950, 36 in 1950-1951, 49 in 1951–1952, 50 in 1952–1953, and 50 in 1953–1954. The total number of samples analyzed was 517, but the number of different sheep used was 144. Many of the sheep from which blood was taken were in the sample groups for two to four seasons.

Blood was taken from the jugular vein into 50-ml. centrifuge tubes, using sodium citrate as an anticoagulant.

Phosphorus and calcium were determined on 8 per cent trichloroacetic acid filtrates of the citrated blood plasma. The phosphorus was determined by a modification of the method of Fiske and Subbarow,[1] in which 2,4-diaminophenol is substituted for 1-amino-2-naphthol-4-sulfonic acid as the reduc-

TABLE 4—Annual Variation in Plasma Phosphorus, Calcium, and Vitamin A, Based on December Sampling, before Dividing into Winter Feeding Groups

Year	Lot	No. of samples	Phosphorus (mg./100 ml.)		Calcium (mg./100 ml.)		Vitamin A (μg./100 ml.)	
			Mean	Range	Mean	Range	Mean	Range
1950	0	9	4.6	3.6–5.9	11.0	10.2–12.9	28	17–36
	1	9	3.9	3.1–5.0	9.9	8.1–11.2	25	22–30
	4	9	4.1	3.6–4.7	10.9	10.0–12.1	29	23–37
	5	9	4.4	3.6–5.6	10.4	9.5–11.6	30	25–34
		36	4.2	3.1–5.9	10.5	8.1–12.9	28	17–37
1951	0	9	4.0	3.2–4.9	8.9	7.8– 9.8	30	16–34
	1	10	4.3	3.3–5.0	8.6	7.3– 9.8	30	17–39
	4	10	4.6	3.9–5.3	9.0	8.1–10.0	30	17–39
	5	10	3.3	2.8–4.6	8.7	7.2– 9.6	33	25–38
	6	10	4.7	3.8–5.3	9.1	8.0–10.2	31	22–37
		49	4.2	2.8–5.3	8.9	7.2–10.2	31	16–39
1952	0	10	3.5	2.8–5.1	8.4	7.8– 9.1	32	27–38
	1	10	3.3	2.8–4.6	8.2	6.8– 9.2	31	25–44
	2	10	3.3	2.2–4.9	8.3	7.4– 9.2	32	24–40
	5	10	3.3	2.2–4.7	8.1	7.2– 9.0	34	24–49
	6	10	3.6	2.4–4.6	8.3	7.8– 9.1	33	28–46
		50	3.4	2.2–5.1	8.3	6.8– 9.2	32	24–49
1953	0	11	4.9	3.8–5.5	10.4	9.4–12.0	32	22–37
	1	10	4.7	4.0–5.3	10.7	8.9–12.1	33	27–46
	2	9	4.6	3.8–5.2	10.8	9.4–12.4	32	25–38
	5	11	4.2	3.2–5.2	10.5	9.7–11.7	33	27–38
	6	9	4.6	3.9–5.1	10.6	8.3–11.5	30	25–37
		50	4.6	3.2–5.5	10.6	8.3–12.4	32	22–46
Means for all samples		185	4.1	2.2–5.9	9.6	7.2–12.9	31	16–49
Standard deviations				0.8		1.3		5.5

ing agent. Calcium was determined, after precipitation as the oxalate and washing, by the colorimetric permanganate method of De Loureiro and Janz.[2] A standard solution containing 10 mg. of calcium and 5 mg. of phosphorus per 100 ml. was carried through the complete procedure with each group of blood samples. This served as a control on reagent and procedural variability.

Vitamin A was determined by mixing 10.0 ml. of plasma with 10.0 ml. of ethanol, adding 15.6 ml. of n-hexane, shaking twenty minutes in the dark, evaporating a 10.0-ml. aliquot of the hexane to dryness *in vacuo*, dissolving the residue in 1.0 ml. of chloroform, and measuring the color within five seconds after adding 9.0 ml. of the Carr-Price antimony trichloride reagent.[3] The Carr-Price reagent was standardized against the Vitamin A Reference Standard of the "Pharmacopeia of the United States."

RESULTS

Table 3 shows the average plasma phosphorus, calcium, and vitamin A for all the sheep sampled, on each of the 12 dates when blood was taken, between April 1, 1950 and Mar. 29, 1954. The range of individual variations is also shown for each date.

Table 4 shows the averages for each lot for the December sampling for each of the four years and the annual averages for the December samplings of all lots. At the time of the December bleeding, all the sheep had been treated alike since about April 1 of the preceding spring. Except for some supplementary feed during lambing, they had been grazing on the range during the whole period.

Table 5 shows a similar tabulation of the data obtained from all the samples taken in March, at the end of the differential feeding of protein supplements.

Table 6 shows the seasonal variation in plasma phosphorus, calcium, and vitamin A, as indicated by the analysis of blood samples from a lot of 50 sheep taken on Nov. 22, 1952; Mar. 17, 1953; July 2, 1953; and Oct. 9, 1953.

Phosphorus.—Reference to table 3 will show that the phosphorus averages for the 12 samplings vary from 3.4 mg. to 5.4 mg. per 100 ml. of plasma with an average for the total of 517 samples of 4.3 mg./100 ml. and a standard deviation of 0.9. The extreme range of individual samples is 2.2 to 7.2 mg./100 ml.

TABLE 5—Annual Variation in Plasma Phosphorus, Calcium, and Vitamin A, Based on March Sampling, at the Close of the Winter Feeding Period

Year	Lot	No. of samples	Phosphorus (mg./100 ml.)		Calcium (mg./100 ml.)		Vitamin A (μg./100 ml.)	
			Mean	Range	Mean	Range	Mean	Range
1950	0	10	4.2	3.3–5.9	9.8	9.2–10.4	20	14–25
	1	10	4.8	3.4–6.1	9.2	8.3– 9.8	22	17–31
	4	10	4.7	3.8–5.4	9.6	8.8–10.0	21	9–31
		30	4.6	3.3–5.9	9.5	8.3–10.4	21	9–31
1951	0	8	5.0	4.2–6.1	9.2	8.8– 9.8	19	13–26
	1	9	5.1	4.0–6.1	8.8	7.3– 9.6	20	12–26
	4	9	5.1	4.0–6.3	9.2	8.6– 9.7	23	17–36
	5	8	4.4	4.0–6.2	9.2	8.5– 9.7	27	19–34
		34	4.9	4.0–6.3	9.1	7.3– 9.7	22	12–36
1952	0	10	3.6	3.2–4.2	9.0	8.0– 9.8	27	20–33
	1	10	3.9	2.9–5.0	8.9	7.6– 9.9	27	20–36
	4	9	3.7	3.2–4.4	9.5	9.0–10.6	27	23–32
	5	10	3.9	3.6–4.6	9.1	8.0– 9.8	31	21–38
	6	10	3.7	3.2–4.2	9.1	8.6– 9.6	28	22–39
		49	3.8	2.9–5.0	9.1	7.6–10.6	28	20–39
1953	0	10	4.0	3.1–4.6	8.3	7.5– 9.4	21	14–27
	1	10	4.6	3.8–7.2	8.3	7.0– 9.4	19	12–28
	2	10	3.5	3.0–4.5	8.6	7.9– 9.6	22	17–28
	5	10	4.0	3.3–4.9	9.0	8.1– 9.4	34	27–48
	6	10	4.2	3.5–5.1	8.5	7.5– 8.9	25	17–35
		50	4.1	3.0–7.2	8.5	7.0– 9.6	24	12–48
1954	0	11	4.6	3.8–5.5	8.6	8.2– 9.1	23	19–27
	1	10	4.9	4.0–6.5	9.1	7.8– 9.9	24	19–30
	2	9	4.2	3.1–5.1	9.4	8.4–10.3	24	17–28
	5	10	3.7	2.7–4.7	8.9	8.2– 9.5	38	32–53
	6	8	4.6	4.0–5.2	9.5	8.6–10.3	25	19–31
		48	4.4	2.7–6.5	9.1	7.8–10.3	27	17–53
Means for all samples		211	4.3	2.7–7.2	9.0	7.0–10.6	24	9–53
Standard deviations				0.8		0.65		6.8

As shown in table 4, the average phosphorus level for the 185 samples taken in December, before the several protein supplement lots were set up, was 4.1 mg./100 ml., with a standard deviation of 0.8, and an individual range of 2.2 mg. to 5.9 mg./100 ml. This table also shows the yearly averages which, for 1950 and 1951, were the same as for the whole period. The average for 1952 was considerably below the general average, while for 1953 it was above the general average.

The average phosphorus level for the total of 211 samples taken in March was 4.3 mg./100 ml., which was the same as the general average for all samples over the whole period and only 0.1 mg. higher than the December average. This indicates that the protein supplements fed to the majority of the sheep did not materially increase the blood phosphorus. This is confirmed by comparing the averages of the different lots for all the March samplings. Lot 0, which received no supplement, shows an average of 4.3 mg., which is higher than three of the supplemented lots.

Because of the seasonal variation in blood phosphorus, it is difficult to establish a "normal." The mean of 4.3 for all the samples reported here is somewhat below a value representing all seasons equally, as only one of the 12 sampling dates was in the early summer season when the blood phosphorus of range sheep is at its peak. The average for the 46 sheep bled in July was 5.4, with a range of 3.5 to 6.9. The only published report on the blood phosphorus of range sheep in the United States is that of Beeson, Terrill, and Bolin.[4] They determined the blood phosphorus of 40 range ewes at the U. S. Sheep Experiment Station at Dubois, Idaho, taking samples at five periods during each year for four years. The averages for the samplings of these ewes varied from 3.81 to 6.16, with a general average for all samplings of 5.09. The period of the highest general average in their ewes was when the sheep were fed concentrate on winter range, while in the Montana work, the sheep fed concentrate did not in general show higher blood phosphorus than those receiving no concentrate.

Unpublished data[5] from Dr. W. T. Hardy, superintendent of the Ranch Experiment Station at Sonora, Texas, involving blood samples taken monthly from four groups of 24 sheep each, grazing on the Edwards Plateau, in the years 1938-1941, gave an average serum phosphorus value of 3.6 mg./100 ml. This is somewhat lower than our corresponding average of 4.3 mg./100 ml. Whether or not the Edwards Plateau is an area deficient in phosphorus was not stated.

TABLE 6—Seasonal Variation in Plasma Phosphorus, Calcium, and Vitamin A

Date	Lot	No. of samples	Phosphorus (mg./100 ml.)		Calcium (mg./100 ml.)		Vitamin A (μg./100 ml.)	
			Mean	Range	Mean	Range	Mean	Range
11-22-52	0	10	3.5	2.8–5.1	8.4	7.8– 9.1	32	27–38
	1	10	3.3	2.8–4.6	8.2	6.8– 9.2	31	25–44
	2	10	3.3	2.2–4.9	8.3	7.4– 9.2	32	24–40
	5	10	3.3	2.8–4.7	8.1	7.2– 9.0	34	24–49
	6	10	3.6	2.4–4.6	8.3	6.8– 9.1	33	28–46
		50	3.4	2.2–5.1	8.3	6.8– 9.2	32	24–49
3-17-53	0	10	4.0	3.1–4.6	8.3	7.5– 9.4	21	14–27
	1	10	4.6	3.8–7.2	8.3	7.0– 9.4	19	12–28
	2	10	3.5	3.0–4.5	8.6	7.5– 9.6	22	17–28
	5	10	4.0	3.3–4.9	9.0	8.1– 9.5	34	27–48
	6	10	4.2	3.5–5.1	8.5	8.2– 8.9	25	17–35
		50	4.1	3.0–7.2	8.5	7.0– 9.6	24	12–48
7-2-53	0	8	5.5	4.4–6.6	9.0	8.3– 9.8	26	22–33
	1	10	5.3	4.4–6.7	8.7	8.1– 9.8	24	20–29
	2	10	5.8	3.5–8.3	9.0	8.1–10.1	25	19–31
	5	10	5.3	4.1–6.0	9.2	8.3–10.0	28	19–39
	6	8	5.3	4.0–7.6	8.7	8.6–10.3	26	23–30
		46	5.4	3.5–8.3	8.9	8.1–10.3	26	19–39
10-9-53	0	6	4.0	2.9–4.5	10.3	9.3–11.4	39	31–46
	1	9	4.3	2.7–6.2	10.1	9.6–10.9	37	29–49
	2	8	4.1	3.3–5.0	10.1	8.7–10.8	37	27–49
	5	10	4.0	3.0–5.4	10.5	9.6–11.4	40	32–48
	6	8	4.0	3.6–5.4	10.4	9.0–11.8	38	30–48
		41	4.0	2.7–6.2	10.3	8.7–11.8	38	27–49

Determinations of blood phosphorus have been made for sheep on native range in Australia and South Africa. Watson,[6] in South Australia, reported averages for sheep on pasture varying from 4.1 to 4.7, while averages of three small groups of sheep on arid range varied from 3.1 to 4.8. In South Africa, Bekker and Rossouw [7] reported that a blood phosphorus level of 6.5 mg./100 ml. is normal for sheep. They reported an average of 3.6 for 79 sheep three weeks after coming off a range low in phosphorus and an average of 2.4 for 5 sheep on a low-phosphorus range. Du Toit, Malan, and Rossouw [8] reported an average of 6.5 for 10 sheep on an experimental ration corresponding to grass containing 0.5 per cent P_2O_5, and 3.8 for 10 sheep on a ration containing 0.25 per cent P_2O_5. Bekker [9] made monthly determinations for nine months on 24 sheep on a pasture low in phosphorus, and obtained averages varying from 3.6 to 6.1, with a mean of 4.4.

Calcium.—As shown in table 3, the calcium averages for the 12 bleeding dates vary from 8.3 mg./100 ml. to 10.6 mg./100 ml., with a general average for the 517 samples of 9.2, a standard deviation of 1.0, and an individual range of 6.8 to 12.9. The data in table 4 show that the December samplings in 1950 and 1953 were definitely higher than in 1951 and 1952, and that the average of all the 185 December samples was higher than that of the March samples. The average for the 211 March samples (table 5) was 9.0, with only slight deviation from this average among the yearly means. As was true for phosphorus, the supplemental feeding did not increase the blood calcium. There was no significant difference among the lots in the lot averages for all the March samplings and, as shown in table 5, there was no significant difference among years. The seasonal picture for the year 1952-1953 (table 6) shows a relatively low value for Nov. 22, 1952, with slight increases in March and July, 1953, and marked increase in October 1953.

We have been unable to find in the literature any information on the normal plasma calcium for sheep on native range. However, in a personal communication,[5] Hardy reports a mean serum calcium of sheep on the Edwards Plateau in Texas of 12.9 mg./100 ml. This value is well above our corresponding one of 9.2. The explanation of this difference is not evident from the information available. In our experience, except for extreme calcium deprivation, plasma calcium levels are not influenced by the calcium content of the feed. The Montana sheep were grazed on a calcareous soil and so should not have been deficient in calcium.

Vitamin A.—As shown in table 3, the means of the vitamin A levels for each of the 12 sampling dates varied from 21 to 38 μg./100 ml., while the mean of all samples was 29 μg./100 ml., with a standard deviation of 7.3, and a range of 12 to 53 μg./100 ml. Table 4 shows that the mean for all December samplings was 31 μg./100 ml., with a standard deviation of 5.5 and a range of 16 to 49 μg./100 ml. This table also shows that the means for the four years show no significant difference, ranging from 28 to 32. There is no significant difference among lot means. The mean of all the March samplings was slightly lower than the December mean, being 24 μg./100 ml. The only significant differences among lots were in the consistently higher vitamin-A level for lot 5, which was wintered on hay, and a slightly higher mean for

TABLE 7—Condensed Comparison of Means for All Samples of All Lots, December, March, and July Samples of All Lots, and Lot 0 Samples

Date	Lot	No. of samples	Phosphorus (mg./100 ml.)		Calcium (mg./100 ml.)		Vitamin A (μg./100 ml.)	
			Mean	s.d.	Mean	s.d.	Mean	s.d.
All dates	All lots	517	4.3	0.9	9.2	1.0	29	7.3
	Lot 0	111	4.3	0.8	9.3	1.0	27	10.7
December	All lots	185	4.1	0.8	9.5	1.3	31	5.5
	Lot 0	39	4.3	0.8	9.7	1.2	31	3.7
March	All lots	211	4.3	0.8	9.0	0.65	24	6.8
	Lot 0	49	4.2	0.7	9.0	0.7	22	4.9
	Lot 5	38	4.0	0.6	9.3	0.5	33	7.1
July	All lots	46	5.4	1.0	8.9	0.6	26	3.7
	Lot 0	8	5.5	0.8	9.0	0.5	26	3.9

s.d.—Standard deviation.

lot 6, which received dehydrated alfalfa pellets.

Two reports by Peirce,[10,11] in Australia, on blood vitamin A in sheep on pasture agree quite closely with our findings. He obtained an average of 25 μg./100 ml. in 18 sheep on dry pasture, with a range of 18-37. In a second article, he reported the results of 29 determinations on 8 sheep throughout the year. The mean was 32 μg./100 ml., with an individual range of 24 to 48. We have not found other reports of vitamin-A determinations for ewes run under conditions similar to those under which our range sheep are handled. Pope, Phillips, and Bohstedt[12] report averages for 18 ewes on pasture in summer and dry lot in winter. For 16 determinations between one month before lambing and 13 weeks after lambing, the averages varied from 37 to 48 μg./100 ml., with an individual range of 17 to 72. Weir, Pope, Phillips, and Bohstedt[13] report averages for 9 ewes on alfalfa hay and grain varying in seven samplings from 24 to 39 μg./100 ml. Colby, Cunha, and Warwick[14] report averages for 44 ewes on hay in dry lots varying from 20 to 26 μg./100 ml.

Discussion

Although the sheep which were the source of the data reported here were in an experiment which involved several nutritional treatments during the winter months, it is considered that the findings as to plasma phosphorus, calcium, and vitamin A may be taken as a basis for "normal" levels under range conditions in the mountain areas of south-central Montana. Reference to table 7 will show that the means for all samples in all the lots were almost identical with the means for all samples from lot 0, which consisted of ewes run on the range throughout the year, with no supplement. The same is true of the December samplings as a group, the March samplings as a group, and the one set of July samples. Table 7 also shows that the only notable difference between the values for lot 5 which was fed hay and concentrate during the winter, and the general values was in the higher vitamin-A level for the March samplings.

It may be concluded that ewes run on the type of range grazed by these sheep may be expected to have the following mean values for plasma phosphorus, calcium, and vitamin A:

Phosphorus	4.1 to 4.3 mg./100 ml., except in early summer when it may be 5.5 mg./100 ml.
Calcium	9.0 to 9.7 mg./100 ml.
Vitamin A	22 to 31 μg./100 ml.

It may also be concluded that feeding of protein concentrates on the range does not increase the phosphorus levels over those for grazing only.

Summary

The means of 517 determinations of levels of phosphorus, calcium, and vitamin A in blood plasma of breeding ewes run on the range in the mountain area of south-central Montana were as follows:

Phosphorus	4.3 mg./100 ml.	(standard deviation 0.9)
Calcium	9.2 mg./100 ml.	(standard deviation 1.0)
Vitamin A	29 μg./100 ml.	(standard deviation 7.3)

The samples were taken in groups of 30 to 52 on 12 dates over a four-year period, 11 of which were between October 9 and April 1. One set of 46 samples taken July 2 showed a higher phosphorus level, namely 5.4 mg./100 ml.

Ewes fed a protein concentrate during the period of pregnancy did not show values significantly different from those for ewes on range with no supplement.

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Protection of Sheep Against Anthrax by Immunization with an Antigen Prepared *in Vitro*

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IMPROVEMENTS in the technique of vaccination of domestic animals against anthrax by the use of an attenuated strain of *Bacillus anthracis* have been announced by Sutton [1] and others. The danger still exists, however, that the weaker and more susceptible animals will succumb to the disease as a result of the inoculation intended to accomplish immunization by Pasteur's method. A safe antigen effective for the protective immunization of these naturally susceptible domestic animals in anthrax enzootic regions would be economically advantageous.

Gladstone [2] reported the production of a protective antigen with which he immunized several laboratory animals and 3 sheep against anthrax. The latter withstood a challenge dose of *B. anthracis* that killed 3 control animals. Watson *et al.*[3] immunized rabbits, guinea pigs, other laboratory animals, and also sheep, with an extract of lesions of rabbits that had died of anthrax. Other than this, very little work of this nature on domestic animals has been reported. Heckly *et al.*[4] and others, including the authors,[5] have prepared antigens free from bacterial cells or spores which successfully immunized guinea pigs and rabbits. Tresselt and Boor [6] have reported the immunization of monkeys with the dried fraction of a cell-free filtrate of a culture of *B. anthracis* similar to that used in the present work.

This report reviews the method of preparation of the antigen and describes its use in the safe and effective protection of sheep against anthrax.

EXPERIMENTAL DATA

Preparation of the Protective Antigen.—The cell-free filtrate of the sixteen- to eighteen-hour culture of *B. anthracis* grown in the medium here described contained the protective antgien. This was concentrated by chemical fractionation, dialyzed, and lyophilized. The product was a stable and convenient form of the antigen suitable for effective immunization of sheep.

The composition per liter of the medium was as follows:

Sheep serum albumin	10.0 Gm., dry wt.
Yeast extract (described below)	5.5 Gm., dry wt.
Sodium bicarbonate	5.0 Gm.
Dextrose	10.0 Gm.
Phosphate buffer, pH 7.0 (1.5 % solids)	700.0 ml.
Phenol red, as pH indicator	12.0 mg.
Water, to make	1,000.0 ml.

The serum albumin chosen was prepared from the species of animal to be immunized to avoid unnecessary introduction of foreign proteins. The yeast extract was prepared by extracting bakers' yeast for twenty-four hours with an hydrochloric acid (HCl) solution at pH 4.0 and then removing from the cell-free filtrate the protein precipitate at pH 11.0. Additional protein, not necessary for the production of the antigen, was removed from the yeast extract by treatment with activated charcoal.

The yeast extract and phosphate buffer were prepared in large quantities and were sterilized by autoclaving at 15 lb. of pressure for twelve to fifteen minutes. The culture medium, pH 7.0, was sterilized by Seitz filtration and distributed in 50-ml. amounts in 12-ounce flat prescription bottles plugged with cotton. It was acidified by absorption of carbon dioxide gas and inoculated with 35,000 spores of *B. anthracis* per bottle. The bottles were capped with parafilm, laid on their sides, and incubated statically at 37 C. for sixteen to eighteen hours.

The bacterial cells were removed by centrifugation and Seitz filtration and the filtrate was fractionated [7] by salting out methods or by alcoholic precipitation at low temperature. The effective protective antigen was shown by rabbit immunization tests to be in that portion of the culture filtrate precipitated as a pseudoglobulin by about 46 per cent saturated ammonium sulfate, or as fraction IV by the alcoholic precipitation method of Cohn *et al.*[8] This fraction, dialyzed at 4 C. and lyophilized, was kept under partial vacuum in the refrigerator.

Immunization of Sheep Against Anthrax.—Immunization by the Usual Method of Administration.—Six Hampshire sheep (709, 710, 79, 70, 72, and 69, table 1) were injected intradermally at six-day intervals with three doses, 20 mg., 40 mg., and 60 mg., respectively, of the protective antigen concentrated by alcoholic precipitation (fraction IV).

As shown in table 1, these immunized animals survived intradermal challenges with 1,200 to 120,000 spores of a virulent

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strain of *B. anthracis*,* administered after intervals ranging from seven to eighty-four days after immunization.

Two sheep (69 and 72) survived without apparent illness the challenge dose of 1,200 spores while the 2 controls (88 and 71) died in ten and nineteen days, respectively, after a similar challenge (*see* section on "Anthrax in Nonimmunized Sheep"). They were challenged again thirteen days later with an intradermal injection of 12,000 spores and were slaughtered twenty-six days after the second challenge.

Necropsy** revealed no lesions of anthrax and the organism could not be demonstrated by bacteriological examination. Animal 79 was slaughtered when it apparently became very ill twelve days after receiving the challenge with 12,000 spores of *B. anthracis*. Instead of anthrax, necropsy revealed a generalized infection with an organism belonging to the genus Clostridium.

Immunization by One Injection of the Antigen.—The "one-shot" method of immunization of sheep, cattle, or other domestic animals obviously would be the most practical system if it would develop a high grade resistance against anthrax. Two sheep (713 and 714, table 1) were injected subcutaneously with 240 mg. of the lyophilized protective antigen in 5.0 ml. of sterile aluminum hydroxide cream.† Sixty-three days later their resistance against anthrax was challenged with 120,000 spores of the same strain of *B. anthracis* used for challenging all of the animals discussed in this report. Twenty days after the challenge, necropsy revealed no pathological changes and no *B. anthracis* organisms were demonstrated by bacteriological examination.

* The resistance against anthrax was determined by challenge with a strain of *B. anthracis*, one or two spores of which injected intradermally in the rabbit or guinea pig established fatal anthrax.

** Three of the control sheep (71, 76, 88) were necropsied by Drs. Joseph Victor, Abou D. Pollock, and Edwin M. Lerner II. Necropsy of the remaining 15 sheep was done by the authors and assistants who are grateful for the valuable service and cooperation rendered by the pathologists named.

† The aluminum hydroxide cream was prepared according to Hawk, Oser, and Summerson.[5]

TABLE 1—Immunization of Sheep Against Anthrax

Immunization doses		Challenge			Second challenge		
Identification	No. of doses *	Days after last dose	Spores *Bacillus anthracis* strain CD25	Days after challenge **	Spores *B. anthracis*	Days after second challenge	Anthrax found
713	1	63	1.2×10^5	20K	.....	.....	neg.
714	1	63	1.2×10^5	20K	.....	.....	neg.
709	3	84	1.2×10^5	20K	.....	.....	neg.
710	3	84	1.2×10^5	20K	.....	.....	neg.
79	3	10	1.2×10^4	12K	.....	.....	neg.
70	3	10	1.2×10^4	25K	.....	.....	neg.
72	3	7	1.2×10^5	13R	1.2×10^4	26K	neg.
69	3	7	1.2×10^5	13R	1.2×10^4	26K	neg.
Controls, Not Immunized							
80	0	....	1.2×10^4	25K	.....	.....	++ †
76	0	....	1.2×10^4	5D	.....	.....	++++
88	0	....	1.2×10^5	10D	.....	.....	++++
71	0	....	1.2×10^5	13R	1.2×10^4	6D	++++†
92	0	....	1.2×10^5	28R	1.2×10^5	20K	+++ †
96	0	....	1.2×10^5	9D	.....	.....	++++
97	0	....	1.2×10^5	7D	.....	.....	++++
717	0	....	1.2×10^5	6D	.....	.....	++++
711	0	....	1.2×10^5	28R	1.2×10^5	20K	neg.
SNO	0	....	1.2×10^5	9D			++++

* 1—One subcutaneous injection of 240 mg. of the antigen (fraction IV, *see* text) in aluminum hydroxide; 3—Three intradermal injections with solutions of 20, 40, and 60 mg. of antigen, respectively.

** K—Destroyed and necropsied; D—died and necropsied; R—sheep 69, 72, and 71 were rechallenged with an intracutaneous injection of 12,000 spores of *Bacillus anthracis* thirteen days after the first challenge; sheep 711 and 92 were rechallenged in like manner with 120,000 spores of this strain twenty-six days after the first challenge.

++ = Severe anthrax, shock, recovery; +++ = severe anthrax, shock, recovery, *Bacillus anthracis* organisms found upon necropsy; ++++ = very severe terminal anthrax, lesions and *Bacillus anthracis* organisms found upon necropsy.

† Sheep 71—Necropsy indicated that the fatal anthrax was the consequence of the first challenge.

Sheep 80—Prostrated, presumably from anthrax, sixth through eighth day after challenge but recovered.

Sheep 92—Appeared to recover from the first challenge; *Bacillus anthracis* was found in spleen, liver, and peritoneal fluid upon necropsy.

Anthrax in Nonimmunized Sheep.— Seven of 10 control sheep died of anthrax after intradermal injection with 120 to 12,000 spores of the virulent strain *B. anthracis* CD-25 (table 1).

Four of the control sheep were injected intradermally in the dorsal scapular region with 120 spores of *B. anthracis;* 3, (97, 717 and SNO) died in seven, six, and nine days, respectively, following severe anthrax; the fourth (711) survived a 28-day observation period.

Four other control animals were challenged in like manner with 1,200 spores. Sheep 96 and 88 died after nine and ten days, respectively. Sheep 71 apparently had recovered thirteen days after the challenge and was given a second injection of 12,000 spores of *B. anthracis* in the thigh. It died six days later, nineteen days after receiving the first challenge of 1,200 spores. At necropsy a small edematous lesion was observed at the site of the second injection. The lesions in the neck and chest anterior to and near the site of the first injection were of such magnitude and severity that one would conclude that the first dose of 1,200 spores was fatal. The outer larynx and the epiglottic folds were severely congested. The fourth control animal (92) that had received the challenge with 1,200 spores recovered after a severe illness. The latter and sheep 711, that had withstood a smaller challenge, were rechallenged with 120,000 spores of this virulent strain of *B. anthracis* twenty-eight days after the first challenge. When no signs of anthrax were observed for twenty days, they were destroyed and necropsied. No anthrax lesions were found in either, but *B. anthracis* organisms were found in the liver and spleen of sheep 92. One (76) of the 2 other control sheep challenged with 12,000 spores died in five days, while the second (80) showed no evidence of anthrax when destroyed after twenty-five days although it had been very ill and prostrated on the sixth, seventh, and eighth days. At necropsy, all of the sheep that had died showed severe hemorrhagic, gelatinous lesions anterior to the site of injection. *Bacillus anthracis* cells were abundant in the spleen, liver, lungs, and peritoneal fluid in these animals which evidently died of severe anthrax.

Discussion

Protection of domestic animals in anthrax enzootic regions is an economic necessity. This can be accomplished effectively by immunization with the protective antigen described.

An abundant, stable antigen was produced in the culture medium described. The yeast extract insured a luxuriant growth of *B. anthracis.* Although the serum albumin was an additional source of culture nutriment, its primary value was its stabilizing effect on the antigen. Inclusion in the culture medium of proteins which might have lead to sensitization of the subject of immunization was avoided. Most of the protein in the yeast extract was removed by precipitation with acid and then with alkali and the remainder was adsorbed on activated charcoal. For the same reason, the serum albumin was selected from the species of animal which was to be immunized. Separation of the antigen from other constituents of the culture by fractional precipitation further insured its freedom from extraneous sensitizing agents.

Protection of man by similar immunization with the safe cell-free antigen in regions and occupations that may bring him in contact with the hazard of infection by *B. anthracis* might become a practical and general procedure which would not be feasible with the attenuated spore-vaccine method of Pasteur.

Summary

The preparation and concentration of protective antigen produced *in vitro* is described. Sheep were effectively protected from anthrax by immunization with the cell-free antigen either by a course of three intradermal injections of an aqueous solution or one subcutaneous injection of the antigen adsorbed on colloidal aluminum hydroxide which acted as a stabilizer or adjuvant.

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Correction

In the Article, "Sheep Pox and Its Control in Egypt Using a Desiccated Live Virus Vaccine," by M. S. Sabban (April, 1955, p. 209), the sheep pox strain used for the preparation of vaccine, which was sent from Persia by Dr. K. Kasteven of the Food and Agriculture Organization, was the Rumanian strain.

Wherever in the article Persian strain appears, it should read Rumanian strain.

Serological Methods for Determining Resistance to Infectious Canine Hepatitis

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THERE IS no standard serological technique for the diagnosis of infectious canine hepatitis (ICH). The test currently used is the complement-fixation test, using infected dog liver as a source of antigen.[1, 9, 11] Supernatant fluids from tissue-cultured virus also have been used.[3]

The conglutinating complement-absorption test has been used less frequently as a serological method for general diagnostic purposes. It has not been previously applied to the routine diagnosis of ICH. The technique has been described in detail as applied to the diagnosis of other infections.[2, 6-8] Its use in the diagnosis of canine distemper has been reported.[4] We were unable to confirm this work.

The present report compares the complement-fixation test and the conglutinating complement-absorption test in serological studies of ICH.

MATERIALS AND METHODS

Serums.—Blood was obtained from unselected street dogs. Serum was removed from the clot and stored at −25 C. prior to use. Control serums were obtained from dogs recovered from experimental infection and dogs proved to be susceptible by subsequent infection.

Antigens.—Infected dog livers were the source of antigen. The animals used for antigen production were 3 to 6 months of age and were susceptible to hepatitis virus as determined by the ICH skin test.[9] Two or more animals were used for the production of antigen. Infection was produced by intravenous injection of 0.5 ml. of a 20 per cent infected liver suspension. When an initial rise in body temperature occurred, usually forty-eight to seventy-two hours after injection, the animals were killed by injection of nembutal.® The abdomen was opened and the entire liver, less the gall bladder, placed in a preweighed Waring blendor. After determining the weight, sufficient buffered saline (pH 7.2) was added to make a 20 per cent suspension of liver. The material was homogenized for five minutes.

The liver suspension was then distributed to 50-ml. centrifuge tubes and centrifuged at 2,000 r.p.m. for thirty minutes at 5 C. The supernatant fluid was distributed in 50-ml. amounts to 1-liter round-bottom flasks, the contents shell frozen, thawed at room temperature, and recentrifuged. The process was repeated a second time and the final supernatant dried from the frozen state in the 1-liter flasks. The dried material was ground in a mortar and stored in brown, screw-top bottles. Antigen titrations were done against positive serum in the usual manner for complement-fixation tests. Four antigen units were used in the test. Fresh antigen was prepared each day by suspending the appropriate amount of dried material (by weight) in saline. Control antigens were prepared as above from normal dog liver.

Complement.—Guinea pig complement was used for complement-fixation tests. Male guinea pigs of approximately 250 to 300 Gm. were bled, and the serum was separated and stored at −70 C. until used.

Cat complement was used in the conglutinating complement-absorption techniques. Normal domestic cats were bled by cardiac puncture. This serum also was stored at −70 C. until used.

Conglutinin.—Normal beef serum was used as the source of conglutinin. Blood was obtained at the time of slaughter and the serum was separated and stored at −25 C. Before use, the serum was inactivated at 56 C. for thirty minutes.

Complement-Fixation Test.—Complement Titration.—Titrations were done in the usual manner in the presence of 0.25 ml. of antigen (4 units). Sensitized sheep cells (0.5 ml.) were added to the complement-saline-antigen mixtures after one-hour incubation at 37 C. The amount of complement causing complete hemolysis was considered 1 unit. Two units of complement were used in the test.

Twofold dilutions of inactivated serum (0.25-ml. amounts) were used in each test group. Antigen (0.25 ml.) was added, followed by 0.5 ml. of guinea pig complement containing 2 hemolyzing units. Appropriate antigen and serum controls were included with each set of tests. All tubes were incubated overnight at 5 C. Sensitized sheep cells (0.5 ml.) were added and incubated fifteen minutes at 37 C. before reading.

Conglutinating Complement-Absorption Test.—Conglutinin Titrations.— A 1:10 dilution of inactivated bovine serum (56 C. for 30 minutes) was used in the titration. Amounts increasing by 0.02 ml. were placed in a series of 15 tubes. The initial tube contained 0.06 ml. of serum. Sufficient saline solution was added to each tube to make a total volume of 0.6 ml. Cat serum, 0.2 ml. of a 1:40 dilution, was added to each tube, followed by 0.2

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TABLE 1—Technique for Complement-Fixation Test for Infectious Canine Hepatitis

Tube No.	Saline solution (ml.)	Control antigen (ml.)	Antigen (ml.)	Serum (ml.) and dilution	Complement (ml.)		Sensitized sheep cells (ml.)	
1	.....	.....	0.25	0.25 of 1:4	0.5	Incubate 24 hours at 5 C.	0.5	Incubate 15 minutes at 37 C. and read.
2	.....	.....	0.25	0.25 of 1:8	0.5		0.5	
3	.....	.....	0.25	0.25 of 1:16	0.5		0.5	
4	.....	.....	0.25	0.25 of 1:32	0.5		0.5	
5	.....	.....	0.25	0.25 of 1:64	0.5		0.5	
6	.....	.....	0.25	0.25 of 1:128	0.5		0.5	
7	.....	.....	0.25	0.25 of 1:256	0.5		0.5	
Control 1	0.25	.....	.....	0.25 of 1:4	0.5		0.5	
Control 2	0.5	.....	.....	.....	0.5		0.5	
Control 3	0.75	.....	.....	.....	....		0.5	
Control 4	0.25	.....	0.25	.....	0.5		0.5	
Control 5	.....	0.25	.....	0.25 of 1:4	0.5		0.5	
Control 6	.....	0.25	.....	0.25 of 1:8	0.5		0.5	
Control 7	0.25	0.25	.....	.....	0.5		0.5	

Control antigen = normal liver suspension; antigen = infected liver suspension.
Note.—Known positive and negative serums were run with each set of test serum to control nonspecificity of reactions.

ml. of a 0.25 per cent suspension of sheep cells. The mixtures were incubated at 37 C. for thirty minutes. All tubes were centrifuged at 1,500 r.p.m. for three minutes. The precipitated material was resuspended by gentle tapping of each tube, and all tubes were allowed to stand at room temperature for one minute before reading.

The tube showing complete aggregation of cells with a clear supernatant fluid was considered as the end point. The amount of serum in this tube was designated as 1 unit. A dilution of serum was made to contain 4 units of bovine serum in each 0.2 ml. Equal volumes of serum dilution and 0.25 per cent sheep erythrocytes constituted the conglutinating system.

Complement Titration.—Amounts increasing by 0.02 ml. of 1:40 cat serum were placed in a series of 15 tubes. The initial amount was 0.06 ml. Sufficient saline solution was added to each tube to make a total volume of 0.4 ml. and 0.2 ml. of antigen dilution (2 units) was added to each tube. After incubation for thirty minutes at 37 C., 0.4 ml. of conglutinating system was added to each tube and the tubes were reincubated at 37 C. for thirty minutes. The tubes were then centrifuged, resuspended, and read as described above. The tube showing complete conglutination contained 1 unit of complement. Two units were used in the test.

The Conglutination Test.—Each serum was serially diluted in 0.2-ml. amounts through ten tubes. Two units of antigen were added, followed by 2 units of complement. Inactivation of test serums was done at 56 C. for thirty minutes, after an initial dilution of 1:4. Known positive and negative serums were used with each test group. After incubation at 37 C. for thirty minutes, 0.4 ml. of conglutinating system were added to each tube. All tubes were reincubated, centrifuged, resuspended, and read. The titer of the serum was interpreted as the serum dilution of the last tube showing no conglutination.

Controls.—In addition to positive and negative serum controls, each serum was examined for anticomplementary activity by placing the lowest dilution in a tube with saline, complement, and the conglutinating system. Each serum was also examined for nonspecific activity against normal dog

TABLE 2—Technique for Conglutinating Complement-Absorption Test for Infectious Canine Hepatitis

Tube No.	Saline (ml.)	Control antigen (ml.)	Antigen (ml.)	Serum (ml.) and dilution	Complement		Sensitized sheep cells (ml.)	
1	....	....	0.2	0.2 of 1:4	0.2	Incubate 1 hour at 37 C.	0.4	Incubate 1 hour at 37 C., centrifuge 3 minutes at 1500 r.p.m., resuspend, and read.
2	....	....	0.2	0.2 of 1:8	0.2		0.4	
3	....	....	0.2	0.2 of 1:16	0.2		0.4	
4	....	....	0.2	0.2 of 1:32	0.2		0.4	
5	....	....	0.2	0.2 of 1:64	0.2		0.4	
6	....	....	0.2	0.2 of 1:128	0.2		0.4	
7	....	....	0.2	0.2 of 1:256	0.2		0.4	
Control 1	0.2	....	....	0.2 of 1:4	0.2		0.4	
Control 2	0.2	....	....	0.2 of 1:8	0.2		0.4	
Control 3	0.2	....	0.2	.....	0.2		0.4	
Control 4	....	0.2	....	0.2 of 1:4	0.2		0.4	
Control 5	....	0.2	....	0.2 of 1:8	0.2		0.4	
Control 6	0.4	....	....	.....	0.2		0.4	
Control 7	0.6	....	....	.....	....		0.4	
Control 8	0.4	0.2	....	.....	0.2		0.4	

Control antigen = normal liver suspension; antigen = infected liver suspension.
Note.—Positive and negative serums are run with each set of test serum.

TABLE 3—Distribution of Reactions of 95 Dog Serums to the Complement-Fixation and Conglutination Tests

Group	Total serums	Conglutinating titer							Complement-fixation titer						
		Negative	1:8	1:16	1:32	1:64	1:128	1:256	Negative	1:8	1:16	1:32	1:64	1:128	1:256
1) Higher complement-fixation titer	24	18	1	5	....	....	....	....	....	6	8	7	3	....	....
2) Higher conglutination titer	26	....	3	5	4	7	6	1	5	5	8	6	2	....	....
3) Equal titers	6	....	....	1	2	3	....	....	....	....	1	2	3	....	....
4) Negative	39	....	....	....	....	....	....	....	....	....	....	....	....	....	....

liver in the lowest dilution of the former. Appropriate antigen, complement, and conglutination system controls were run with each test.

RESULTS

The standard procedures for the two tests used in the studies are outlined in tables 1 and 2.

The distribution of reactions of 95 canine serums to both tests is shown in table 3. Twenty-four dogs showed a higher complement-fixation titer than conglutinating complement-absorption titer. Eighteen (75 %) of those serums, however, were negative to the latter test. In group 2, 26 serums showed a higher conglutinating complement-absorption titer. Only five serums (19.2 %) were negative to the complement-fixation test.

Thirty-nine serums were negative to both tests. Among the 56 positive serums, 33 showed reactions to both tests. Of the latter number, 21 (63.6 %) showed a higher conglutinating titer, whereas only six (18.1 %) demonstrated a higher complement-fixation titer.

Of 17 dogs showing negative serums to both tests, 35.3 per cent were positive to the ICH skin test. Eleven dogs positive to the conglutination test were all positive to the skin test. Four animals that showed positive complement-fixation but negative conglutination titers were also skin tested. Three were negative and 1 was positive.

DISCUSSION

For an efficient serological procedure, it is necessary that a standardized technique be employed. Accordingly, it is proposed that the method outlined here, or a similar procedure, be adopted for the serological identification of ICH. Concerning a preferred test, it is probable that the complement-fixation system will be used until additional information is available on the adaptability of the conglutinating complement-absorption technique in this specific instance.

The source of antigen for routine use may be improved. It is possible that the method of tissue culture of ICH virus, recently described by Cabasso *et al.*[3] and Fieldsteel and Emery,[5] may yield an improved antigen. A central source of antigen for all laboratories also would be advantageous.

These limited trials comparing the two serological procedures can only be used to draw preliminary conclusions. It does seem obvious, however, that when both tests show a positive reaction, the conglutinating complement-absorption titer is most often highest. A relatively high percentage of serums, however, show a positive complement-fixation titer, but a negative reaction to the other method.

It may be assumed, therefore, that nonspecific reactions occur with frequency in the complement-fixation test and that the conglutination technique is both more specific and more sensitive. Additional investigation is necessary before these factors can be accurately determined.

SUMMARY

Serological identification of antibodies to infectious canine hepatitis (ICH) virus is discussed. Standard procedures for the complement-fixation and conglutinating complement-absorption techniques are outlined. The reactions of 95 serums to both tests were studied. Of serums showing reactions to both tests, 63.6 per cent showed a higher conglutinating complement-ab-

sorption titer, and 18.1 per cent demonstrated a higher complement-fixation titer.

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Effect of Desoxycorticosterone Acetate on Young Chicks

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DESOXYCORTICOSTERONE acetate (DCA) administered daily to young Leghorn chicks caused gross tissue changes, especially in the testes. The testes were enormously enlarged and a single layer of epithelium was found lining the seminiferous tubules. These changes were attributed to an accumulation of fluid in the testes caused by the DCA. There was also an accumulation of serous-like fluid in the peritoneal cavity.[5] Involution of the seminal vesicles and testes in mature and immature rats is caused by DCA. Leydig cells and spermatogenic tubules are affected.[4] Under the influence of DCA, rats voluntarily drink more water and urine output is enhanced, while urinary sodium and chloride are much reduced in concentration and total amount excreted.[1] The amount of sodium chloride in the diet influences the development of lesions resulting from continued administration of DCA.[6]

The object of this study was to observe changes in chicks caused by continued DCA administration and the effect of posterior pituitary hormone in the disturbed water balance.

EXPERIMENTAL

Ninety White Plymouth Rock male chicks, 4 days of age, were divided into three groups. One group of 70 chicks received two subcutaneous injections of 0.5 mg. of DCA in 0.05 ml. of mazola oil daily for seven days. The dose was then reduced to 0.25 mg. twice a day for the remaining twenty-three days. A group of 10 birds received injections of 0.05 ml. of mazola oil daily. The remaining 10 birds served as untreated controls. All birds were fed the same ration which contained 1 per cent sodium chloride, and were maintained under similar conditions.

In a second part of the experiment, 18 birds which had received DCA for twenty days and had an excessive amount of fluid in the peritoneal cavity were divided into two groups of 9 birds each. Birds in both groups continued to receive 0.5 mg. of DCA daily. The birds in one group were also given 0.5 U.S.P. unit posterior pituitary hormone intramuscularly daily for ten days.

Eight treated birds (none of those used in the second part of the experiment) and 4 control birds were destroyed at the conclusion of the thirty-day injection period. The testes, heart, spleen, kidneys, liver, pancreas, and adrenals were removed and weighed. These organs were fixed in Zenker's solution and sectioned for histological study. Laparotomies were performed on the remaining 38 treated birds and a few of the controls. The testes were observed and their approximate length recorded.

Because of the severe macroscopic and microscopic damage to the testes by DCA, it became of interest to see if the testicular components retained their ability to respond to gonadotrophic hormones. For this reason, 16 chicks which had received DCA for thirty days were treated with sheep pituitary at the rate of 5 Gm. equivalent (in terms of acetone-dried anterior pituitary powder) per bird in eight days. Injections were begun three days after termination of the DCA treatment. Body weight and comb measurement (expressed as product of length times height) were made on all birds at weekly intervals during the experiment.

RESULTS

Desoxycorticosterone acetate had a pronounced effect on the birds. On the sixth day of treatment, 1 chick died after developing extensive edema. Seventy-nine per cent of the chicks developed edema during the treatment. Therefore, a reduction in the amount of the hormone administered seemed advisable. Twenty-four treated chicks died during the injection period presumably from the toxic action of DCA. All chicks that died had an excessive amount of fluid in the peritoneal cavity (table 1).

The treated chicks appeared to eat more than the controls and their average weight was greater (graph 1). Part of this weight was due to fluid retained in the body and was not due to true growth. Examination revealed very little fat in the bodies in the chicks.

TABLE 1—Effect of Desoxycorticosterone Acetate (DCA) on Young Chicks During Thirty-Day Injection Period

	Number of Chicks			
Treatment	1st day	30th day	Developed edema	Died
DCA	70	46	55	24
Mazola oil	10	10	0	0
Untreated	10	10	0	0

From the Department of Veterinary Physiology and Pharmacology (Link), Department of Animal Science (Nalbandov), and Agricultural Experiment Station, University of Illinois, Urbana.

TABLE 2—Body and Organ Weights (Gm.) of Normal and Desoxycorticosterone Acetate-Treated Chicks 34 Days of Age

	No. of chicks	Body wt.	Kidneys	Liver	Heart	Testes	Adrenals
Treated	8	302	2.92	9.01	2.54	0.121*	0.026
Untreated	4	289	2.20	7.04	1.58	0.044	0.024

* Fluid was drained from testes before weighing.

The kidneys were enlarged and their surfaces irregular. There was mild tubular hypertrophy and enlargement of the glomeruli. Slight thickening of the walls of the kidney arteries was noted.

There was marked increase in the size of the hearts and histological examination revealed hypertrophy of the ventricular muscle fibers.

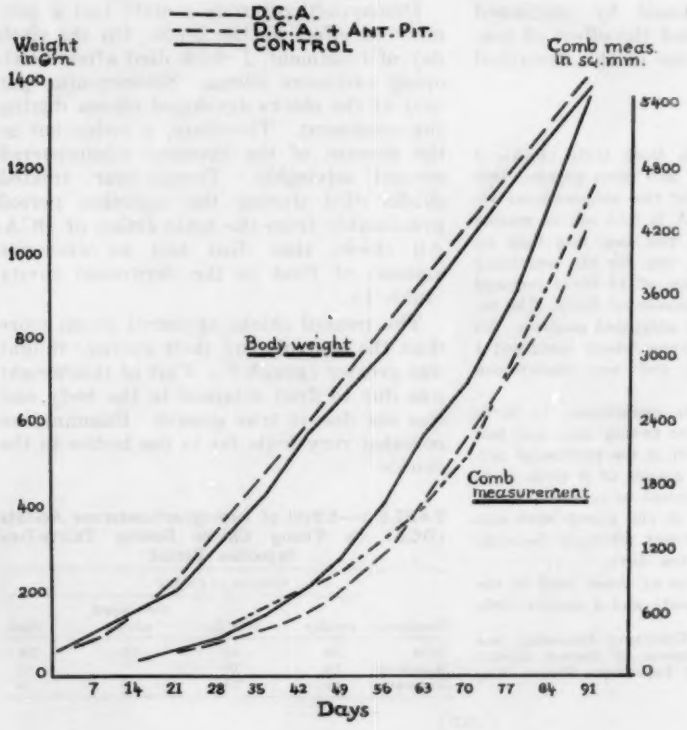
The livers were enlarged. The enlargement appeared to be due to hypertrophy of the liver cells and to passive hyperemia, although there was no evidence of hemorrhage (table 2).

The pancreas showed no change other than slight edema.

There was atrophy of the cortical tissue but no significant change in total weight of the adrenals. Birds which were maintained until 90 days of age (60 days after treatment) showed almost complete recovery from the adrenal atrophy. The livers and hearts also appeared normal at that age.

Of the 46 treated birds living at the end of the injection period, 28 (61 %) had testes which were approximately four times the length of the controls, 6 (13 %) had testes twice the length of the controls, and those of the remaining 12 (26 %) appeared normal in size.

The testicular enlargement appeared to be due primarily to accumulation of fluid in the spermatogenic tubules. The tubules were lined by flattened epithelial cells and showed no signs of spermatogenic activity. Leydig cells appeared normal. There was extensive edema in the interstitial tissue and numerous enlarged veins and capillar-



Graph 1—The effect of desoxycorticosterone acetate on body weight and comb growth of male chicks.

ies. The arteries and veins in the tunica vasculosa were distended (fig. 1, A).

In the second part of the experiment, the birds which were treated with posterior pituitary showed marked reduction in the generalized edema. Only 1 bird of this group of 9 died. Eight of the 9 similar birds not treated with posterior pituitary died. Examination of the 8 birds treated with posterior pituitary hormone at the conclusion of the thirty-day DCA injection period revealed that only 3 (37 %) of the 8 birds had enlarged edematous testes. Size of the testes at the beginning of the period

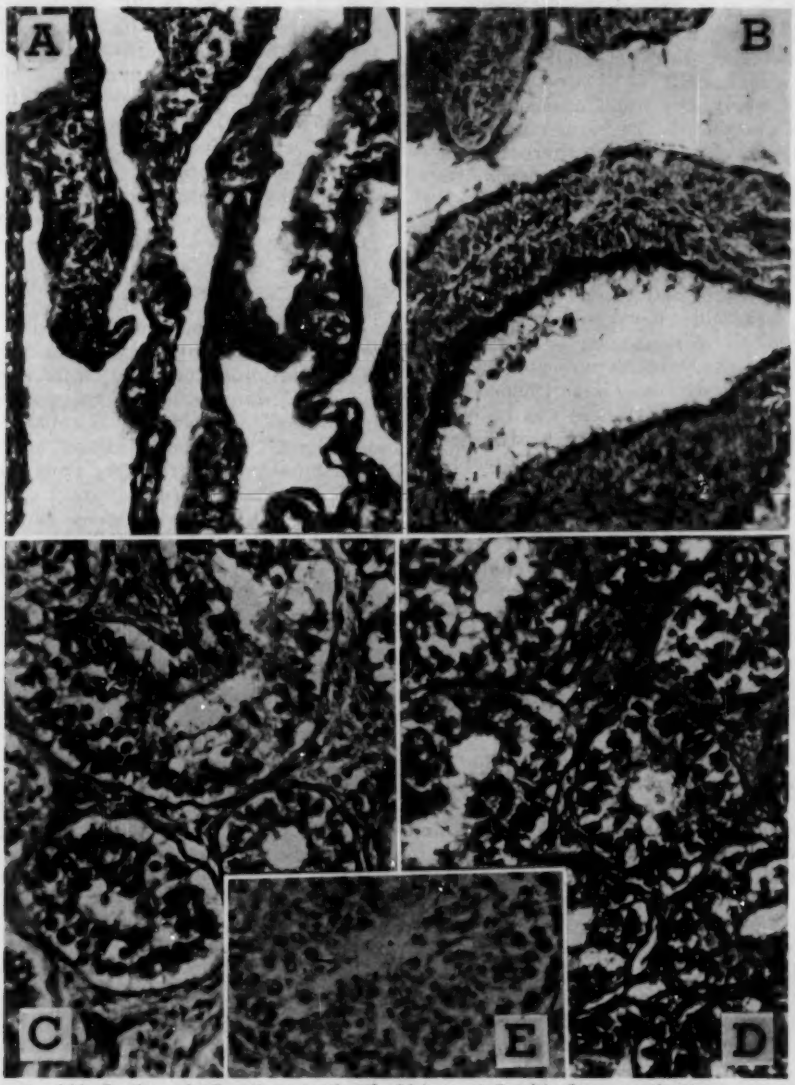


Fig. 1—(A) Section of edematous testis of chick treated with desoxycorticosterone acetate (DCA) for thirty days. (B and C) Similar testes sixty days later, (C) stimulated with gonadotrophic hormones. (D) Testis (60 days after DCA) which was normal in size at conclusion of DCA treatment. (E) Testis from control chick (94 days of age). Hematoxylin and eosin stain. x 400.

during which posterior pituitary hormone was injected was not determined because the chicks did not appear strong enough to withstand laparotomies.

Chickens used in the third part of the experiment were examined when 94 days of age. These birds had been injected with sheep pituitary from the thirty-third to forty-first day. The response of the enormously enlarged testes of the birds treated with anterior pituitary following discontinuation of DCA is illustrated in figure 1, C. Grossly, the testes appeared normal in size and shape. There was no evidence of sperm production but there was more tubular development than in similar testes which were not stimulated (fig. 1, B).

The response of the normal-sized testes to anterior pituitary in the 6 DCA-treated birds is illustrated in figure I, D. There was less interstitial tissue than in figure 1, C, and more development of tubular cells but no spermatogenesis. Androgenic activity of the testes as indicated by comb growth suggests some impairment of this function (graph 1). There was no observable difference between the birds injected with mazola oil and the untreated controls.

DISCUSSION

Continued administration of DCA to young chicks caused marked changes in many tissues. Generalized edema was extensive. Desoxycorticosterone acetate favors the retention of sodium and is known to correct the disturbed electrolyte balance in Addison's disease without significantly altering the disturbed water balance.[2, 8] The antidiuretic hormone of the pituitary inhibits the reabsorption of sodium and chloride but increases the reabsorption of water.

Evidence was presented by Silvette and Britton[7] suggesting a physiological antagonism between the diuretic action of adrenal cortical hormone and the antidiuretic action of posterior pituitary hormone. However, Sartorius and Roberts[3] concluded that DCA does not have direct action on water reabsorption nor an antagonistic action to pitressin® antidiuresis. Renal changes in the chicks did not appear sufficiently extensive to explain the extensive edema. It appears possible that the increased reabsorption of salt results in increased reabsorption of water and favors the development of extensive edema observed in many of the chicks.

The permanent injury to the germinal epithelium of the testes may be due to a specific action of DCA. Injury to this structure was evident in the testes which were not edematous. Therefore, this effect does not appear to be due entirely to pressure from the edema fluid. There was little evidence of repair in this tissue sixty days after discontinuation of DCA. Anterior pituitary hormones did not restore normal structure in the edematous testes when the birds were 90 days of age suggesting that the effect of DCA is persistent and that this effect is direct and not due to suppression of the anterior pituitary. Atrophy of the adrenals appeared to be a compensatory process and, after withdrawal of DCA, there was almost complete recovery from this effect.

SUMMARY

The subcutaneous administration of desoxycorticosterone acetate (DCA) to young chicks had profound effect on many organs. It caused damage to the germinal epithelium of the testes which was apparent sixty days later. The heart, kidneys, and adrenals showed marked recovery from the effect of DCA after a period of sixty days. Androgenic function of the testes, as indicated by comb growth, was slightly impaired. Posterior pituitary reduced the edema caused by DCA.

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The Relative Importance of Body Temperature and Lymphocytes in Genetic Resistance to *Salmonella Pullorum* in the Fowl

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PROOF that chicks may differ genetically in ability to resist infection with *Salmonella pullorum* is provided by the fact that resistance can be increased by selection [18] and also by the significant differences among breeds in susceptibility.[10]

Concerning the physiological basis for such resistance, there have been differing views. From previous studies at this laboratory, Scholes and Hutt [22] concluded that it results from better control of the thermoregulatory mechanism by the resistant chicks. In their experiments, body temperature rose more rapidly in resistant chicks than in susceptible ones during the first ten days after hatching. Experimental lowering of body temperature caused a corresponding lessening of resistance, whereas induction of artificial fever increased it. Chicks of a resistant breed (White Leghorns) showed at 2 to 10 days of age consistently higher temperatures than chicks of a susceptible breed (Rhode Island Reds). Moreover, among sibling chicks from different Rhode Island Red dams, those with mean familial temperature above the average were more resistant than those with familial temperatures below the average.

Subsequently, Severens, Roberts, and Card [20] concluded that resistance and susceptibility to *S. pullorum* were dependent upon the number of lymphocytes in the blood. These were more numerous in their resistant strain, increased concurrently with resistance during the first few days after hatching, and seemed to do so more rapidly in resistant chicks than in susceptible ones. Reduction in the number of lymphocytes following x-ray irradiation was associated with a decrease in resistance. Although Roberts *et al.*[20] also found body temperatures to be higher in the resistant strain than in the susceptible one, that difference was not considered important.

Since in these two independent studies, one group of investigators attributed resistance to body temperature and the other to lymphocytes, it seemed desirable to determine (1) any relationship that might exist between body temperatures and lymphocytes, and (2) any relationship between resistance and (*a*) each of these factors separately, or (*b*) both together. Results of such investigations and of others related to them are reported herewith.

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MATERIALS AND GENERAL PROCEDURES

Stock.—The chicks used in most of these experiments were offspring of White Leghorn hens mated with F_1 males from a cross of White Leghorn ♂ ♂ with Rhode Island Red and New Hampshire ♀ ♀. In some experiments pure White Leghorns and Rhode Island Reds were also used. The parent stocks were all free from pullorum disease, as was demonstrated by agglutination tests.

Management.—All chicks were wing-banded at hatching and identified as to sire and dam. They were then transferred to the specially designed, thermostatically controlled brooders used by Scholes and Hutt [22] in which temperatures were held to variations within 1 C. (fig. 1). The chicks were disturbed only once a day when feed was added to the hoppers, body temperatures recorded, and the brooder cleaned. They were usually kept in these brooders for their first ten days, after which inoculated chicks were transferred to battery brooders for a further period of ten days. Mortality was recorded to 21 days of age. All brooders and equipment were thoroughly cleaned and disinfected before each experiment.

Culture.—Throughout these studies only one strain of *S. pullorum* was used. This culture, which had been recently isolated from a young

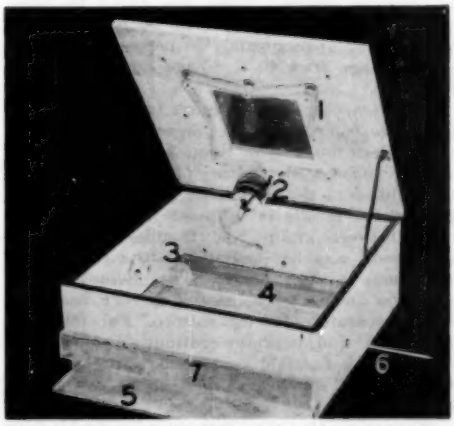


Fig. 1—Type of brooder used with (1) heating coil, (2) thermostat, (3) lamp, (4) wire floor of ⅓-inch mesh, (5) sliding metal floor pan (6) thermometer, and (7) hinged door.

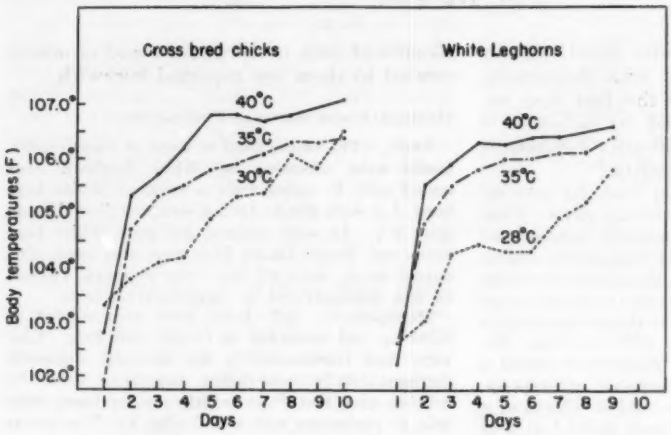
chick that died of natural infection, was maintained by frequent transfer to fresh blood-agar slants and later to nutrient-agar slants every two weeks.

Dosage.—Chicks were inoculated individually *per os* with a 24-hour, nutrient-broth culture by means of a 1-ml. glass syringe attached by a rubber tube to a 4-inch narrow, thick-glass tube. The inoculum was introduced directly into the crop. The dose was always 0.4 ml. of whatever dilution was used.

Since the maximum information concerning resistance and susceptibility is determined when mortality is around the level of 50 per cent, various dilutions were tried to determine which would induce that result. After several trials, a dilution of 1:50 in distilled water was found to cause (with the particular strain of *S. pullorum* and chicks used) approximately 50 per cent mortality, with a range from 40 to 60 per cent in different lots.

Variation.—As is usual, some variation was observed in mortality from one experiment to another. Such fluctuations result from variability in the environment, in virulence of the pathogen, and in condition of the host. They prevent direct comparisons of results in different trials but differences between groups and families within each experiment may be compared with corresponding differences in other similar trials. Because of the variation among trials, results in each one were tested separately for significance. For this purpose the χ^2 (adjusted for continuity) method was used with a fourfold contingency table showing numbers that survived or died in the two groups being compared.

Diagnosis.—Mortality was recorded to 21 days of age. Every chick that died was examined for characteristic gross lesions and also by culture. The cultures were made from the liver on nutrient-agar plates and incubated for twenty-four hours at 37 C. In addition to observation of the characteristic colonies of *S. pullorum*, confirmation was made by the agglutination test, using a known immune serum. This was adopted as a regular routine for identification. The serum was obtained from a rabbit after immunization with the same strain of *S. pullorum*, following the method of Edwards and Bruner.[4]



Graph 1—Effects of brooder temperatures (Centigrade) on body temperatures (Fahrenheit) of chicks 1 to 10 days of age.

RESISTANCE AND TEMPERATURE

Recording Temperatures.—Chick temperatures were taken with a rectal mercury thermometer after insertion through the cloaca for one minute, following the technique of Lamoreux and Hutt.[14] Insertion was facilitated by dipping the bulb in mineral oil. Uniform depth of insertion was ensured by a stopper made of adhesive tape wound several times around the thermometer at a point 5/8 inch from the tip of the bulb. Further to ensure uniformity, all temperatures were taken by one of us (T.R.) between 12:30 and 4 p.m. each day which, according to Hilden and Stenback,[7] Fronda,[6] and Lamoreux and Hutt [14] is the period of peak diurnal temperature. Variation was further minimized by taking only 1 chick at a time from the brooder, which otherwise was kept closed.

Normal Temperatures.—As the first step, temperatures from hatching to 10 days of age were taken of 225 pedigreed crossbred chicks, not inoculated, and brooded at 35 C., which is the normal brooding temperature for that age. At the same time temperatures were taken of 22 crossbred chicks

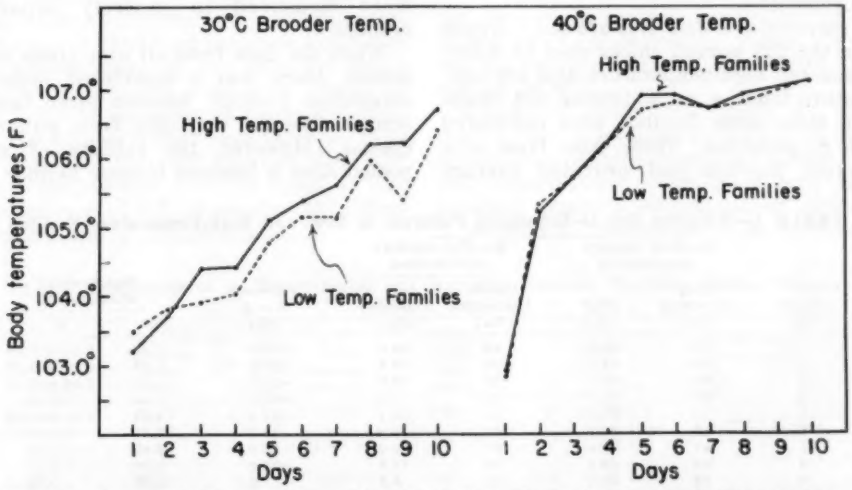
brooded at 30 C., of another 22 at 40 C., and of three lots of White Leghorns (22 per lot) brooded at 28, 35, and 40 C. (graph 1).

On removal from the incubator, the crossbred chicks had temperatures ranging from 96.5 to 105.8 F., the mean figure for 225 being 101.9 ± 0.13 F. In chicks brooded at 35 or 40 C., temperatures rose rapidly during the first five days, reaching 105.8 F., respectively, on the fifth day. Thereafter, the rise continued at a much slower rate to ten days when mean temperatures were 106.4 and 107.1 F., for the groups brooded at 35 and 40 C., respectively. In chicks brooded at 28 and 30 C., body temperatures rose more slowly during the first five days and did not reach normal temperature until the tenth day. Their lag below normal was particularly evident during the first six days. Statistical tests showed that both in crossbreds and in White Leghorns the mean body temperature of the chicks brooded above or below the normal 35 C. deviated significantly from the means for those at 35 C.

Altogether these results show that chicks are in a partly homoiothermic state when hatched, that their thermoregulatory mechanism functions poorly in the first few days, and that it gradually becomes stabilized by about ten days. These findings confirm similar evidence adduced by Lamoreux and Hutt [14] and Scholes and Hutt.[22]

Interfamilial Differences in Temperature.—To ascertain whether or not there were differences among families in mean body temperature during the first five days after hatching, familial averages were determined for two sire families and 22 dam families comprising the 225 crossbred chicks that were not inoculated. These chicks were hatched in different lots over a period of three months and brooded at the normal 35 C. Since they were all brooded under identical conditions and there were no significant differences among different lots, the temperatures of all lots were grouped together to determine family averages. Each chick's temperature was recorded daily; the average number of chicks per dam was originally 10.2, and the actual average number of temperature readings in five days per dam's family was 49.4.

The mean body temperature of these 225 chicks during the first 5 days was 104.6 ± 1 F. Averages for the 22 families ranged from 103.6 to 105.3 F. Mean temperatures in the two sire families were 104.4 and 104.9 F., the difference being significant at the level $P = <0.05$. An analysis of variance showed that there were highly significant differences ($P = <0.01$) among the 22 dam families, and also among the dam



Graph 2—Average body temperatures at 1 to 10 days of age for the ten high-temperature families and 12 low-temperature families, showing difference at 30 C. (brooder temperature) which is obliterated at 40 C.

families within each sire family. Similar significant familial differences in body temperature of Rhode Island Red chicks were found by Scholes and Hutt.[22]

As the number of chicks in any one dam's family was rather small for interfamilial comparisons of temperature and of viability, the 22 families were divided into two classes. One of these comprised ten families in which the average one- to five-day temperature was above the mean for the whole group (104.6 F); the other comprised the remaining 12 families, all below that mean. For these high-temperature and low-temperature groups, the mean temperatures were 105 and 104.4 F., respectively.

After this division of the families had been made according to their temperatures up to 5 days of age, further analysis showed that the difference between the two groups was maintained consistently right up to 10 days of age. In other representatives of the two groups kept at the subnormal brooder temperature of 30 C., the difference was also consistently maintained to 10 days but, when samples of the two groups were brooded at 40 C., the genetic difference between them was obliterated (graph 2). The high brooder temperature apparently provided for the low-temperature families an environment in which they could maintain body temperature at a normal level.

Temperature and Resistance.—Apart from the 225 normal chicks used to differentiate the high-temperature and low-temperature families, an additional 621 chicks from these same families were inoculated with *S. pullorum*. These came from nine different hatches and provided average samples of 28 chicks from each of the 22 families. The dosage was 0.4 ml. of a 1:50 dilution of a 24-hour broth culture given six to seven hours after removal of the chicks from the incubator. Some hatches were given other dilutions or dosed at slightly later ages, but all chicks of any one hatch received identical treatment. These inoculated chicks were all brooded at 35 C. for the first ten days and were then transferred to a battery brooder (with heated and unheated compartments available) where they were kept until 21 days old. Chicks from high-temperature families and from low-temperature families were intermingled during each trial.

In all nine tests, mortality of the inoculated chicks was consistently lower in the high-temperature families than in those with temperatures below average (table 1). Even with the comparatively small numbers involved in any one experiment, the difference was statistically significant ($P = <0.03$) in five of the nine trials. For all tests combined, the cumulative χ^2 showed the difference to be significant at the level of $P = <0.001$.

The superior resistance of the high temperature families thus proved is all the more significant because the interfamilial (genetic) differences in temperature were determined in certain samples from each family, while the differences in resistance were measured in entirely separate samples.

When the data from all nine trials were pooled, there was a significant negative correlation (−0.49) between mean family temperature and mortality from pullorum disease. However, the validity of such pooled data is lessened because neither the

TABLE 1—Mortality Due to Salmonella Pullorum in Low- and High-Temperature Families

Hatch No.	A—Low-temperature families		B—High-temperature families		Difference B − A	Significance of difference	
	Chicks inoculated (No.)	Died (%)	Chicks inoculated (No.)	Died (%)	(%)	χ^2	P
1	26	69.2	25	44.0	−25.2	2.36	0.10 to 0.20
2	28	82.1	30	56.6	−25.5	3.28	0.05 to 0.10
3	63	92.0	30	76.6	−15.4	3.03	0.05 to 0.10
4	28	35.7	35	8.6	−27.1	5.44	<0.02
5	10	90.0	23	52.1	−37.9	2.44	0.10 to 0.20
6	12	83.3	30	30.0	−53.3	7.81	<0.01
7	43	79.0	61	54.0	−25.0	5.82	<0.02
8	26	46.1	24	12.5	−33.6	5.22	<0.03
9	66	25.7	61	8.2	−17.5	5.65	<0.02
Totals	302	(Ave. 67.01)	319	(Ave. 38.07)		41.05	<0.001
				(Degrees of freedom: 9)			

mortality nor the numbers of chicks from each family were equal in each test. Error from these causes can be overcome to a considerable extent by combining families into larger groups. When this is done, it is clear that familial resistance to *S. pullorum* increases with familial body temperature (table 2).

Differences in Sire Families.—All of the 621 chicks that were inoculated came from 2 sires, A and B. In their normal uninoculated chicks, the mean body temperatures (1 to 5 days) were 104.9 and 104.4 F., respectively, and corresponding mortality in all chicks inoculated as 35.8 and 57.3 per cent, respectively. These figures confirm the evidence from the 22 dams' families that resistance is associated with the higher temperatures. However, since A sired seven of the ten dams' families in the high-temperature group and B sired ten of the 12 dams' families that made up the low-temperature group, it was possible that the striking differences between them in resistance (table 1) could have resulted from some paternal influence other than that of temperature.

To elucidate this point, each sire's dam-families were separated into classes with familial body temperatures above or below the mean for all chicks from the sire. Temperatures and mortality in the four groups thus established are shown in table 3. These show clearly that when the influence of the sire is thus held constant, resistance and susceptibility in the different dam's families within sires is directly related to their mean temperature at one to five days. For the high-sire, high-dam families, mortality at 31.7 per cent was less than half that in the low-sire, low-dam families. The cumulative χ^2 of 9.77 shows that the differences are highly significant.

Effect of Changing Body Temperature upon Resistance.—Since, as the foregoing data show, ability to resist *S. pullorum* is closely related to the chick's body temperature during the first five days after hatching, experimental modification of chick temperatures should cause corresponding variations in resistance. Tests for such confirmative evidence were made by inoculating different lots of chicks which were brooded for their first ten days at high or low brooder temperatures. As was shown earlier, such treatment causes corresponding changes in body temperature. After ten days, all chicks were brooded together in battery brooders and mortality was recorded to 3 weeks of age.

TABLE 2—Differences in Body Temperature and Corresponding Death Rates in Three Groups of Dams' Families

Temperature class	Families (No.)	Average body temperature (F.)	Chicks inoculated (No.)	Mortality (%)
High	7	105.1	233	33.0
Medium	9	104.6	253	57.7
Low	6	104.2	135	61.5

Two such tests were run, each with inoculated controls brooded at the optimum 35 C., and also with noninoculated controls brooded at the low temperature. In the first trial, the low temperature used was 28 C. Since that might have been too low for some of the controls not inoculated (as shown by 9.1% mortality among them), the low temperature for the second trial was set at 30 C.

Results of these tests (table 4) show clearly that experimental raising of body temperature heightens ability to resist and that experimental lowering of body temperature causes a corresponding decrease in resistance. Pathogenicity of the dosage

TABLE 3—Differences in Temperatures and Mortality Between Families Within Progeny of Each Sire

Sire	Dam's families: Temperature class	No.	Ave. body temperature (F.)	Chicks inoculated (No.)	Died: No.	Died: (%)	Significance of difference: χ^2	P
A (high temperature)	High	5	105.2	145	45	31.7	2.312	0.14
	Low	4	104.5	87	37	42.5		
B (low temperature)	High	7	104.7	260	136	52.3	7.465	<0.01
	Low	6	104.2	129	87	67.4		
							9.777	<0.01

was obviously much greater in experiment 1 than in the other. It proved fatal to all chicks brooded at 28 C., but only to 10 per cent of those brooded at 40 C. In experiment 2, with lighter dosage, resistance of the chicks brooded at 30 C. was clearly reduced, while those subjected to experimental hyperthermia were completely resistant. Similar results were obtained by Scholes and Hutt.[22]

TABLE 4—Effect of Lowering and Raising Body Temperatures upon Resistance

Experiment	Body temperature	Brooder temperature (C.)	Treatment	Chicks (No.)	Mortality to 3 wk. (%)
1	Lowered	28	Noninoculated	22	9.1
	Lowered	28	Inoculated	24	100.0
	Normal	35	Inoculated	37	40.5
	Raised	40	Inoculated	20	10.1
2	Lowered	30	Noninoculated	20	0.0
	Lowered	30	Inoculated	25	28.0
	Normal	35	Inoculated	22	9.1
	Raised	40	Inoculated	23	0.0

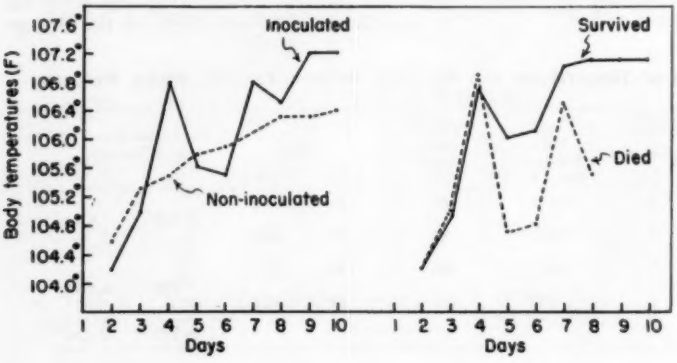
Temperature Changes Following Inoculation.—To determine the response of body temperatures to infection with *S. pullorum*, daily temperatures were taken from 2 to 10 days of age of 36 chicks that had been inoculated seven hours after removal from the incubator. All were brooded at the optimum temperature of 35 C. for the first ten days and, thereafter, were kept in battery brooders to 3 weeks of age.

A comparison of their average daily temperatures with those of normal controls not inoculated (graph 3) shows that infection causes a febrile reaction three to four days after inoculation. Mean body temperature at the peak of this fever was 106.8 F., compared with 105.5 F. in controls of the same age. This peak is followed by relapse to subnormal temperatures and a second rise which is sustained from seven to ten days.

Temperatures of the inoculated chicks varied much more than those of normal controls, the standard errors in the two groups being ± 0.27 and ± 0.05, respectively, at 5 days of age. It seems probable that these variations in temperature five days after inoculation are directly related to resistance and susceptibility. As graph 3 shows, inoculated chicks that later succumbed showed an initial rise in response to infection practically the same as that in chicks which survived. However, temperatures of the susceptible chicks fell next day to about 104.6 F., which was 1.4 F. lower than the figure for chicks that survived. Thereafter, a second attempt to regain higher temperatures was successful in chicks that survived, but much less so in chicks that succumbed.

In each of two other similar trials, temperatures were taken of 15 chicks to which inoculations with *S. pullorum* were given at 2 and 3 days of age. Among these, the course of temperatures following inoculation was much the same as that shown in graph 3, except that in chicks inoculated at 3 days mean body temperatures remained at a lower level than in chicks inoculated at younger ages.

Altogether these findings, which are similar to those of Kelly and Dearstyne [12] and Scholes and Hutt,[22] suggest that resistance to *S. pullorum* is associated with superior control of the thermoregulatory mechanism. In chicks not inoculated, that superiority is shown by more rapid rise of temperatures after hatching. In chicks that are infected, those that can regain and



Graph 3—Body temperatures at 1 to 10 days of age showing febrile reaction in inoculated chicks, and inability of susceptible chicks to maintain temperatures.

maintain high body temperatures survive, but those that can not do so succumb.

RESISTANCE AND BLOOD CELLS

Techniques for Counting Cells.—Because the number of leukocytes per milliliter is much higher in birds than in mammals, a pipette for counting erythrocytes was used for counts of both erythrocytes and leukocytes. Blood was obtained from the vena basilica in the wing, diluted 1:200, and the pipettes were then clamped and kept for several hours in a refrigerator to ensure adequate staining. Rees-Ecker's solution, as recommended by Wintrobe [26] for platelets, provided staining satisfactory for direct count of total leukocytes.

For differential counts, 200 leukocytes were counted in different fields of blood smears stained with Wright's stain. Since it was to the lymphocytes that Severens *et al.*[23] attributed resistance to *S. pullorum*, each leukocyte was classified either as a lymphocyte or not. It was sometimes difficult to differentiate between large lymphocytes and monocytes, but as many as possible of the latter were identified by their comparatively large size, greater amount of cytoplasm and bluish staining.

Total Cell Counts.—At 1, 5, and 11 days of age, average cell counts per milliliter in 23 to 27 normal crossbred chicks were 2.95, 2.71, and 2.85 millions, respectively, for erythrocytes and 13.2, 16.9, and 14.2 thousands for leukocytes. Although there was no significant change with age up to 11 days, there was wide variation in counts of erythrocytes among different chicks on the same day and from day to day. The counts given for these cells and for leukocytes agree well with those found by Cook and Dearstyne [2] and Olson.[16]

Differential Counts.—The proportion of lymphocytes rose steadily from one to eight days (table 5). These findings agree with those of Olson [16] and of Severens *et al.*[23] Since the lymphocytes increase with age, it follows that the proportion of other leukocytes must be correspondingly decreased.

To obtain the data shown in table 5, differential counts were made on 128 chicks from 2 sires and 20 dams. Not all were done each day. The number of chicks per dam varied only from 5 to 7, the average being 6.4. Mean proportions of lymphocytes per family were computed from the counts on the second, fourth, and sixth days. Thus, the family average was based on 15 to 21 determinations per dam's family and ten times these figures for sires' families. Statistical analyses showed that there were no significant differences between the 2 sires or among the 20 dams with respect to the average lymphocyte percentages in their offspring. Similarly, the average lymphocyte percentage for families classified as high-temperature families did not differ from that for low-temperature families. It was 36.5 in both groups.

TABLE 5—Average Lymphocyte Counts of Uninoculated Crossbred Chicks From 1 to 10 Days of Age, Expressed as Proportion of Total Leukocytes

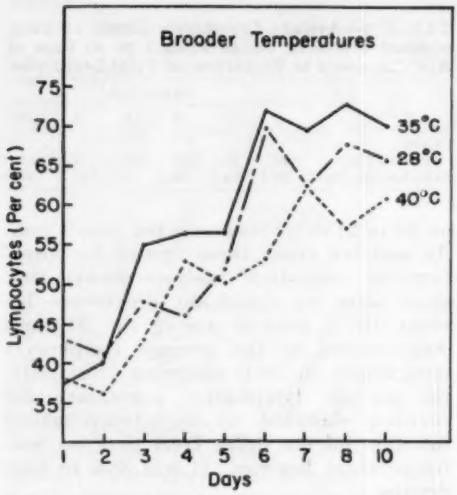
	Days of age					
	1	2	4	6	8	10
Chicks studied (No.)	40	85	122	122	72	121
Lymphocytes (%)	21.6	24.3	34.3	50.3	60.7	57.9

Lymphocytes and Resistance.—Correlation between the lymphocyte count (%) in normal chicks and the mortality from *S. pullorum* in inoculated chicks of these 20 families was found to be only $r = +0.014$. This indicates that there is no relation between lymphocytes and resistance. However, because the numbers of chicks per family varied somewhat in the tests of resistance, it was considered desirable to group several families at each extreme of the range in lymphocytes in order to reduce any possible errors from each source. When the eight families ranking highest in lymphocytes (average, 38%) were compared with the eight lowest (average, 34.9 %), mortality in inoculated chicks of the former group was 44.5 per cent, and in the latter one 45.7 per cent. No relation of lymphocytes to resistance is evident.

Lymphocytes and Body Temperatures.—Since, as the foregoing studies have shown, both the proportion of lymphocytes and body temperature rise during the first eight days after hatching, it seemed possible that there might be a direct relationship between these two variables. In that case both lymphocytes (as claimed by Severens *et al.*[23]) and temperatures (as claimed by Scholes and Hutt [22]) could be

responsible for the concurrent rise in resistance to *S. pullorum*.

To elucidate this point, the data were analyzed to determine whether or not these



Graph 4—Lymphocyte counts at 1 to 10 days of age, showing depression in chicks brooded at low or high temperatures.

two factors were related in families or in individual chicks. Among 20 dams' families the correlation (in chicks not inoculated) between mean body temperature in the first five days, and mean proportion of lymphocytes during the first six days was $r = +0.19$. In 40 chicks, comprising 2 picked at random from each family, the corresponding correlation was $r = +0.20$.

These low coefficients of correlation indicate that there is only very slight relationship, if any at all, between body temperature and proportion of lymphocytes in normal chicks.

Effect of Changing the Body Temperature upon Lymphocyte Count.—To determine the effects of abnormal body temperatures on the proportion of lymphocytes, 66 White Leghorn chicks, 1-day-old, were distributed at random in three lots and brooded at 28, 35, and 40 C. Body temperatures of these chicks (graph 1) show that in those brooded below or above the optimum 35 C. body temperatures were correspondingly subnormal or above normal. Blood smears were prepared daily for ten days from 11 chicks in each group, but each chick was used only on alternate days to give it a 48-hour rest between blood tests.

As graph 4 shows, in chicks brooded at 28 or 40 C., lymphocyte counts were consistently below those for chicks brooded at the optimum 35 C. In chicks subjected to hyperthermia, the proportion of lymphocytes was depressed farther below normal than in chicks brooded at subnormal temperature and having correspondingly subnormal body temperatures. The adverse effect of high temperature was also evident in the slower growth of chicks brooded at 40 C. In these, the mean weight at ten days was 67.2 Gm. against 73.6 Gm. for chicks kept at normal temperature. The difference is highly significant ($t = 2.89$; $P = <0.01$).

It is important to note that although experimentally induced hyperthermia depresses the lymphocyte count (graph 4) and retards growth, nevertheless it does raise the resistance of chicks to *S. pullorum* (table 4).

Blood Cells of Inoculated Chicks.—Following inoculation on the day of removal from the incubator, 30 infected chicks showed a decrease in total erythrocytes from 3.04 million per milliliter on the second day to 2.62 million at eleven days. During this same period total leukocytes increased from 13.9 thousand per milliliter at two days to 33.5 and 36.5 thousand at seven and eleven days, respectively.

Since these latter counts are at least twice the normal figures, the marked increase in leukocytes is clearly one response of the mechanism for defense against infection. Similar leukocytosis following Salmonella infection was observed by Cook and Dearstyne,[2] Kelly and Dearstyne,[12] Taylor,[24] and Olson and Goetchius.[17]

Differential counts on these same 30 chicks showed the proportions of lymphocytes at two, seven, and eleven days to be 22.5, 31.0, and 51.5 per cent, respectively. Since these percentages are somewhat lower than in uninoculated chicks at the same ages (table 5), the cells responsible for the rise in leukocytes in response to infection are probably the heterophils. Similar relative decrease in lymphocytes and increase in heterophils was observed in chicks infected with *S. pullorum* by Kelly and Dearstyne [12] and, following infection with *Salmonella gallinarum*, by Cook and

Dearstyne.[2] In turkeys an increase of heterophils after pullorum infection was noted by Wai and Stafseth.[25] In our chicks the granular structure of the heterophils changed from the fairly uniform rodlike structures found in normal heterophils to small rods, or to large and small round crystalloids.

THE SPLEEN AND RESISTANCE

The belief of Severns, Roberts, and Card [23] that the number of lymphocytes is the chief factor affecting resistance to pullorum infection was apparently confirmed by the fact that the spleen, which is an important source of lymphocytes, was larger in their resistant stock than in their susceptible strain. However, since the former were White Leghorns and the latter Rhode Island Reds, the difference between them in size of spleen is evidently a difference between breeds and, hence, no more significant *per se* in relation to resistance than any other difference between White Leghorns and Rhode Island Reds.

To determine whether or not spleens differed in resistant and susceptible families of the same stock, weight of that organ at 4 days of age was determined in 19 dams' families, each of which provided 5 chicks. These were the same crossbred families that had been used for most of these experiments. In 50 chicks from ten high-temperature families (previously proved resistant) and 45 chicks from nine low-temperature families (previously proved susceptible), the mean weights of the spleen were 45.2 and 40.4 mg., respectively. Since spleen weight was found to be correlated with body weight ($r = +0.31$), the organ weights were regressed on body weights, after which mean spleen weights in these two groups were 44.7 and 41.7 mg. This difference is not significant.

In these 19 families, the correlation between weight of spleen and percentage of lymphocytes was $r = -0.27$. This negative correlation suggests an inverse relationship, rather than a direct one, between spleen size and number of lymphocytes. The correlation between weight of spleen and mortality from *S. pullorum* in inoculated chicks of these same 19 families was $r = -0.18$, which is too low to have much significance, if any. Altogether these data fail to show any importance of the spleen as a factor in resistance to *S. pullorum*.

BREED DIFFERENCES IN RESISTANCE

It was shown in earlier studies that White Leghorns are more resistant to *S. pullorum* than are Rhode Island Reds.[10] However, since both Hutt [8] and Severens *et al.*[23] found that in White Leghorns the proportion of lymphocytes was higher than in the Rhode Island Reds, it seemed desirable to measure in different breeds both lymphocytes and resistance.

The mean daily lymphocyte counts from 1 to 10 days of age for 11 White Leghorns, 120 Rhode Island Reds, and 30 crossbreds were consistently highest in the White Leghorns (graph 5). It was surprising, however, to find that counts in crossbreds were mostly lower than for Rhode Island Reds. As stated earlier, these crossbreds were ¾ White Leghorn, and ¼ Rhode Island Red or New Hampshire.

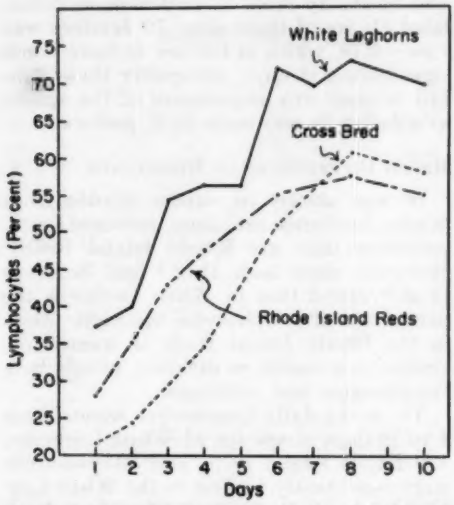
Chicks from these three stocks were inoculated in three different experiments and carried to 3 weeks of age to measure their resistance to *S. pullorum* (table 6). In the first trial the culture was so highly virulent that mortality reached 87 per cent in both groups and completely obliterated any difference in susceptibility. Experiment 2 showed that the crossbreds were much more resistant than the Rohde Island Reds. This was confirmed in experiment 3, which showed a highly significant difference between these two and yielded the additional information that the cross-

TABLE 6 — Breed Differences in Resistance to Salmonella Pullorum

Experiment	Breed	Chicks inoculated (No.)	Mortality		Significance of difference	
			No.	(%)	χ^2	P
1	Crossbred	54	47	87.0	.....	.....
	Rhode Island Red	33	29	88.0		
2	Crossbred	10	2	20.0	2.23	0.10
	Rhode Island Red	24	15	62.5		
3	White Leghorn	53	3	5.7	7.578	<0.01
	Crossbred	50	3	6.0		
	Rhode Island Red	49	14	29.6		

breds were practically as resistant as the Leghorns.

Since the resistance shown by the crossbreds in comparison with the White Leghorns and Rhode Island Reds (table 6) bears no relation whatever to the lymphocyte counts in these three breeds (graph 5), it is impossible to ascribe to the lymphocytes any special role in resistance to *S. pullorum*.



Graph 5—Lymphocyte counts in two breeds and crossbreds at 1 to 10 days of age; all brooded at the normal 35 C.

TEMPERATURE, METABOLISM, AND THE NATURE OF RESISTANCE

To the best of our knowledge, the thermal death point for *S. pullorum* has not been determined. The fact that Lamoreux[13] found some hens that reacted to the agglutination test for this organism to have temperatures over 109 F. suggests that the thermal death point must be still higher. Clearly the relatively high resistance associated with mean chick temperatures around 105 F. for the first five days can not be attributed to destruction of invading bacteria by high temperatures alone.

It was suggested by Scholes and Hutt[22] that higher temperatures are associated with higher rates of metabolism and, thus, with acceleration of defense mechanisms, particularly phagocytosis. According to DuBois,[3] metabolism in man follows fairly well van't Hoff's law for chemical reactions, which holds that each rise in temperature of 10 C. causes a doubling of activity. Moreover, Fenn[5] has shown that the rate of phagocytosis is almost a logarithmic function of temperature.

Such an interpretation is supported by the fact that White Leghorns, which are more resistant to *S. pullorum* than Rhode Island Reds, have higher basal metabolic rates than the latter breed.[15] To test this association without complications caused by other differences between breeds, basal metabolic rates were determined for 72 chicks from our high-temperature (resistant) families, and 72 from the low-temperature (susceptible) families, all chicks being thus of the same crossbred stock.

For these tests a modified Haldane apparatus was used. The period of actual measurement of gas exchange (2 hours) was preceded by a preliminary half hour of confinement in which no records were made. The rate of ventilation was 5 liters per minute. Environmental temperature was 27 C. in the first trial and 25 C. in the second, both being in the zone of thermic neutrality. Production of CO_2 was determined directly from the gain in weight of the CO_2 absorber (NaOH). Consumption of O_2 was measured indirectly by gain in weight of the water and CO_2 absorbers, less the loss in weight of the chicks.

In trial 1, 48 chicks from each group were tested (all 48 together) when 5 days old, after fasting for five hours. Since 48 chicks crowded the chamber somewhat, in trial 2 only 24 chicks of each group were tested, and the fasting period was extended to fourteen hours. Results (table 7) were almost the same in both trials. Heat pro-

TABLE 7—Differences in Basal Metabolism of Chicks from High- and Low-Temperature Families at 5 Days of Age

Type of family	Chicks (No.)	CO_2 given off (Gm.)	O_2 consumed (Gm.)	Heat production* calories
Trial 1				
High temperature	48	12.10	9.75	8.29
Low temperature	48	15.80	8.35	6.96
Differences		−3.70	+1.40	+1.33
Trial 2				
High temperature	24	5.86	6.55	9.86
Low temperature	24	5.33	5.00	8.16
Differences		+0.53	+1.55	+1.70

* Per hour, per kilogram.

duction by chicks of the high-temperature families, when compared with those of low-temperature families, was greater by 19.1 and 20.8 per cent in trials 1 and 2, respectively.

Although it is difficult to say whether high body temperatures (and the superior control of the thermoregulatory mechanism which they indicate) are the cause or the result of the greater metabolic activity of high-temperature families, it is clear that both are associated with resistance to *S. pullorum*. Presumably, that resistance results from acceleration of phagocytosis, bacteriolysis, or production of antibodies at the higher temperatures.

In similar studies with *S. gallinarum*, Bell [1] also found his resistant strains to be characterized by higher temperatures and better control of temperature than were chicks of a susceptible strain. However, since his resistant strains were White Leghorns and their descendants while his susceptible strain was New Hampshires, his results may have been confounded by physiological differences between these breeds. This applies also to his conclusion that polymorphonuclear leukocytes in the resistant strain were better able to digest bacteria than the same cells in the susceptible strain.

DISCUSSION

In support of their claim that resistance to *S. pullorum* depends on the number of lymphocytes, Roberts *et al.*[20] subjected chicks to x-ray treatments and thereby lowered both the number of lymphocytes and resistance. Similar experiments made by Scholes,[21] using a dosage slightly less severe, led him to conclude that any reduction of resistance by such treatment could as well be attributed to the lesions thus induced in the liver and intestines as to anything else. Temperatures of x-rayed chicks were also reduced.

Subsequently, Roberts *et al.*[19] again lowered lymphocyte counts and resistance by x-ray treatment, reaffirmed their belief that these cells are the important factor in resistance, and concluded that there is no relationship between temperature and resistance. The lesions found by Scholes were not mentioned.

The attempt of Roberts *et al.* to relate temperature of a chick on 1 of its first 3 days to its resistance is not justified because body temperature of a chick varies greatly from day to day at that age. Average temperatures from five daily readings give some idea of the chick's thermoregulatory ability but one reading does not. Moreover, the body temperatures recorded by them on the second and third days were abnormally low (102.97 and 103.72 F.) which suggests that their brooding conditions (or the recording) were unsatisfactory. (Corresponding figures for normal chicks in the present study were 104.6 and 105.3 F.) In spite of this, when their chicks were classified according to average temperatures during the first three days, then divided into two groups, one above average (31 chicks in each breed) and one below (29 chicks in each breed), mortality to twenty-one days in inoculated chicks was as follows:

	Temperatures	
	Above average	Below average
White Leghorns	3.2%	10.4%
New Hampshires	44.8%	48.2%

These results, though limited in scope, are in accord with our own conclusion that resistance is greater in the chicks with higher temperatures.

Unfortunately, the conclusions of the Illinois workers about differences between resistant strains and susceptible ones have to be discounted considerably because their resistant strains were White Leghorns but their susceptible one was either Rhode Island Red [23] or New Hampshire.[19] Apart from the more obvious differences between White Leghorns and these heavier breeds in form and color, there are several important physiological differences as well.[11] Among these, at least two are related to the present problem. Adult White Leghorns are more resistant to extreme heat [9] and, in chicks of that breed, body temperatures rise more quickly after hatching.[14, 22] The White Leghorns clearly have better control of the thermoregulatory mechanism than the heavy breeds. As the studies reported in this paper show, that difference is much more important than the breed difference in lymphocytes on which the Illinois workers concentrated their attention.

SUMMARY

Body temperatures and counts of blood cells were recorded daily from 1 to 10 days

of age for 225 normal crossbred chicks from 2 sires and 22 dams, all brooded at the optimum temperature of 35 C. Temperatures rose rapidly during the first five days to a mean of 105.8 F. and, thereafter, more slowly to 106.4 F. at ten days.

Significant differences were found between the 2 sires and among the 22 dams in mean temperature of their chicks at one to five days. Ten high-temperature families were differentiated from 12 low-temperature families as being above or below the one- to five-day mean of 104.6 F. for the whole group. Other samples from the high-temperature families maintained consistently higher body temperatures than chicks of the low-temperature families when both lots were brooded at 30 C., but this genetic difference between the groups was obliterated in chicks brooded at 40 C.

When 621 additional chicks from these families were inoculated with *Salmonella pullorum*, mortality was consistently and significantly lower in chicks of the ten high-temperature families than in the others. Among 145 chicks from the sire and dams that had produced high-temperature families, mortality was 31.7 per cent but, in 129 chicks from low-sire, low-dam families, mortality was 67.4 per cent. The negative correlation between familial temperature of normal chicks and mortality of their inoculated siblings ($r = -0.49$) was significant and fairly high.

Susceptibility of inoculated chicks was increased when they were brooded at low temperature (30 C.) but was consistently reduced in chicks subjected to hyperthermia by being brooded at 40 C.

Among inoculated chicks, those that survived maintained temperatures higher than did those that died, following an initial fever in both groups.

Familial differences in lymphocytes (%) were small, statistically insignificant, and unrelated to mortality of inoculated chicks ($r = +0.014$). Brooding chicks at 40 C. depressed lymphocyte count but raised both body temperature and resistance. In chicks brooded at 28 C., lymphocyte counts were depressed somewhat less but temperatures and resistance were much reduced.

Following inoculation, total leukocytes increased to more than twice the normal figure but, since the proportion of lymphocytes fell below normal, the cells that increased in response to infection were more likely to have been the heterophils. No significant relation could be detected between size of spleen and resistance.

In comparisons of breeds, White Leghorns had consistently higher lymphocyte counts (%) and much greater resistance to *S. pullorum* than Rhode Island Reds. However, crossbred chicks (¾ Leghorn) had lymphocyte counts even lower than in Rhode Island Reds but were equal to the White Leghorns in resistance.

Metabolism tests showed the heat production of chicks from the high-temperature families to be greater by about 20 per cent than that of chicks from low-temperature families.

It is concluded that high body temperatures at one to five days and the superior thermoregulatory control associated with them accelerated defense mechanisms such as phagocytosis, bacteriolysis, and the production of antibodies.

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Quantitative Aspects of Newcastle Disease Virus Infection—Effect of Route of Infection on the Susceptibility of Chicks

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It is generally believed that Newcastle disease (ND) is primarily a respiratory disease and as such is mainly transmitted through the air.[6, 24] Experimentally, chickens may be infected by intranasal or intratracheal instillation [4a, 13, 15] or by allowing the birds to breath a Newcastle disease virus (NDV) aerosol.[2, 14, 16, 21, 23] In several instances, epizootics were reported from farms where no other contact than through the air could be demonstrated. It is also known that birds may be infected through the alimentary route, although seldom has simultaneous respiratory infection been excluded. During an early period of the disease the virus may be found in the intestinal tract,[15] in feces,[10, 29] and in respiratory mucus.[30]

Which route of infection, the alimentary or the respiratory, is more important under natural conditions is still a matter of conjecture. The experiments to be described in this paper throw some additional light on the quantitative aspects of infection by various routes and on the epizootiology of the disease in general.

Materials and Methods

Virus.—A highly pathogenic strain of NDV (designated "HP") was used in this study. It was isolated during an epizootic in 1950 and carried since through approximately 50 passages in embryonating eggs. When tested in 1952 on a group of 7- to 14-day-old susceptible chicks, it caused 90 per cent mortality on infection by contact. The pathogenicity of this strain remained the same in the present series of experiments. On one occasion (experiment 1, table 3) avirulent, Komarov's Haifa vaccine virus [18, 19] was used. The virus was harvested as allantoic fluid from infected embryos, forty-eight to seventy-two hours after inoculation, and stored in a refrigerator at 4 to 6 C. for a period not exceeding one month. During this period the titer of the virus did not change appreciably. For use, the infected allantoic fluid was diluted in phosphate buffer M/15 of pH = 6.8 — 7.1, or in distilled water.

Chickens.—The chickens were hatched in a laboratory incubator from eggs obtained from commercial flocks which had been vaccinated against ND. According to Bornstein,[4] such chicks exhibit partial passive immunity during the first three weeks of their life. With two exceptions, the chicks employed in this study were 3 or more weeks of age.

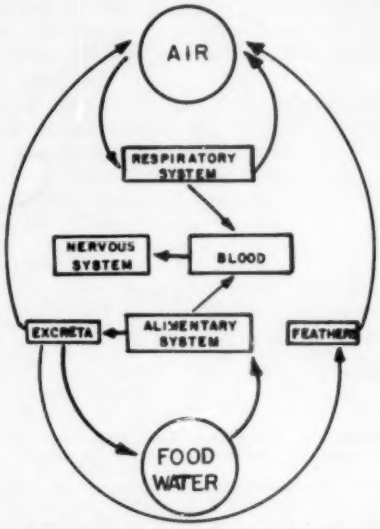

Fig. 1—Infection cycles of Newcastle disease virus in nature.

Cages.—After infection, each chick was housed in a separate wire cage with individual feed and water supply. The animal room was irradiated by four ultraviolet lamps (40 μw./1 sq. cm. at a distance of 1 m.).* The birds were protected from direct radiation by cardboard covers on top of the cages. The bottom of each cage was provided with a removable tray filled with lysol. Spilled food and droppings fell directly into lysol. This arrangement was found to be satisfactory and no cross infections were observed in control chicks held in the same room and under identical conditions.

Intramuscular Exposure.—The virus was in-

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* Sterilamp, WL 782-30, Westinghouse Electric Corp.

jected into the pectoral muscle, next to the sternal septum in a 0.1-ml. volume.

Alimentary Exposure.—For alimentary infection, the virus suspension was introduced directly into the crop by means of a thin polyethylene tubing attached to a syringe. More diluted suspensions were introduced before less diluted ones and the tubing was well wiped each time with an alcohol-soaked cotton pledget. This method of administration of the virus into the alimentary tract was chosen in order to prevent contamination of the respiratory passages from the oral cavity (this was reported as possible when chickens were fed Salmonella-infected food or water, directly or by a pipette inserted into the mouth).[20] The volume of NDV suspension administered into the crop was 0.5 or 1.0 ml.

Intranasal Exposure.—For intranasal inoculation, the chicks were anesthetized with ether until disappearance of the swallowing reflex, and then virus suspensions (0.1 or 0.2 ml.) were dropped into the nostrils by means of a syringe equipped with a blunt needle.

Intratracheal Exposure.—The chicks were anesthetized with ether as described above. The beak was opened and the virus suspension (0.1 ml.) was introduced through the laryngeal slit into the trachea by means of a bent, blunt hypodermic needle.

Aerosol Infection.—Equipment for Exposure.—For satisfactory aerosol infection a special chamber was constructed, based mainly on the design of Weiss and Segeler[21] and Gogolak.[9] The apparatus is diagramatically shown in figure 2. The virus suspension was nebulized by means of a reflux nebulizer (N in fig. 2) as described by Ferry *et al.*[7] This nebulizer was found to produce an aerosol of droplets with an average diameter of 5 to 10 μ. The nebulizer sprayed 0.25 ml. of suspension in thirty minutes at 200 mm. Hg pressure and 1.8 liters per minute of air flow. The aerosol was mixed by means of a Venturi arrangement with air of desired humidity and let through a wide-bore (25 mm.), inverted-U, glass tube into the exposure chamber (E). At the flow rate employed, the aerosol reached the chamber in two seconds. This time was more than sufficient for a complete drying of the droplets to droplet nuclei[12] at a relative humidity of less than 75 per cent. The size of droplet nuclei, consisting of dried virus and suspending salts, was calculated not to have exceeded 1 μ. This size of aerosol particles is known to be maximally retained in mammalian lungs.

The exposure chamber was made of a cylindrical glass jar of 12-liter capacity and large enough to hold 6 chickens comfortably. A circular, aluminum-top plate was sealed to the jar by means of four hinged and butterfly-nut screwed rods. From the exposure chamber, the aerosol was drawn either through an impinger (IM) at the rate of 8.8 to 9.2 liters per minute or was led through a bypass at the same rate of flow to a resin-wool filter† and a flow meter‡ to a vacuum pump. The relative humidity of the air in the chamber was measured by means of dry and wet bulb thermometers (HM) inserted in the bypass. The whole apparatus was held at a negative pressure of 40 to 80 mm. water, as indicated on a manometer

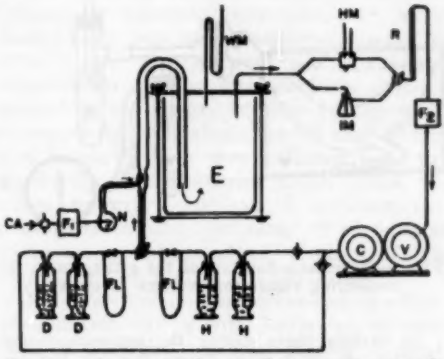


Fig. 2 — Apparatus for respiratory exposure of chicks to Newcastle disease virus.

(WM) connected to the exposure chamber. The diluting air (7 to 7.4 liters per minute) was obtained from a separate compressor which blew the air in two streams, one passing through silica gel and NaOH pellets (D,D) and the other through two gas-washing bottles filled with water (H,H). By combination of varying proportions of these two streams, the desired humidity could be obtained.

Methods of Exposure.—Chickens were introduced into the chamber, the lid closed, and the diluting air was passed through at the rate of 9 liters per minute. The pressure inside the chamber was adjusted to —80 mm. of water and nebulization of virus suspension begun. At the same time, a stop watch was started. Necessary adjustments were made in the flow of the diluting air in order to equilibrate the total flow at a negative pressure. The nebulization continued for twenty to thirty minutes; it was experimentally found that the cloud concentration practically reached its maximum value one and one-half minutes after beginning of nebulization. At the end of the exposure period, the nebulization was stopped and the chamber was ventilated for fifteen minutes at 15 liters per minute of air flow. This rate of ventilation resulted in complete clearing of the chamber air of the nebulized virus. The chamber was then opened, the exposed chickens were removed to their individual cages and the glass jar was cleaned and wiped with alcohol. A new batch of chickens was placed in the dry chamber to be exposed to a higher concentration of virus.

The chickens could be observed in the chamber throughout the experiment. Usually, they moved

† Cape Asbestos Co., London, W. 1, England.
‡ Fisher and Porter, Hatboro, Conn.

about for a few minutes after their introduction into the chamber but soon, having found the most comfortable position, remained quiet for the duration of their exposure. Spraying of virus was not started until the chickens became quiet.

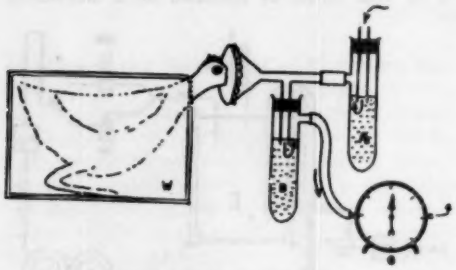


Fig. 3—Schematic diagram of the arrangement for measuring respiratory volume of chicks.

At various times during the exposure period, samples of cloud were drawn through bubbler-impingers containing buffered gelatine solution.[8] Usually two, five-minute samples were taken during each exposure period. The suspension in the impinger was then treated with 5 μg. streptomycin per 1 ml. of suspension [28] and titrated for its NDV content in embryonating eggs in three- and five-fold dilution steps. The concentration of NDV per liter of air (Cv) was then calculated (Reed and Muench method [22]) from the dilution reciprocal (D) at which 50 per cent of the embryos died ($e.l.d._{50}$), the known volume of air which passed through the impinger (Va), the efficiency of the impinger (E), the volume of the collection fluid in it (Vf), and the volume of suspension inoculated (Vi):

$$Cv\ (\text{in } e.l.d._{50}) = \frac{Vf \times D \times 100}{Vi \times Va \times E}$$

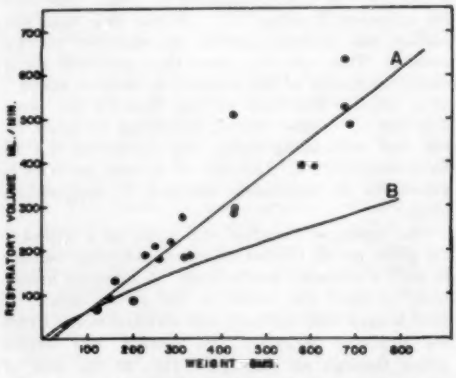
When the concentration of NDV in the air (Cv) was determined as above, the amount of virus inhaled by the chicks was calculated as: dose inhaled (in $e.l.d._{50}$) = T x Rv x Cv; where T was the time of exposure in minutes and Rv the respiratory volume of chicks in liters per minute.

Determination of the Respiratory Volume of Chicks.—The respiratory volume of chicks was experimentally determined in the following way. Chickens of weights varying between 100 and 800 Gm. were put in a wooden box (W in fig. 3) so that only their heads protruded. The beak was pushed to above the nostrils through a slit in a rubber membrane into a glass funnel. The funnel was connected to two test tubes as shown in the diagram. The expired air, having passed through tube B, was led to a gasometer which measured its volume in a given period of time (it was shown that either inspired or expired air could be measured in this way without any difference in the results). Test tube A acted as a one-way valve for the inspired air and test tube B served a similar purpose for the expired air; in each, the inlet and outlet tube was dipped approximately 2 to 3 mm. in water, so that resistance to flow of air was negligible.

Before determinations of respiratory volume were made, the chickens were held in position in the box until quiet and until their breathing became regular. Readings of the volumes of air breathed by the chicken were then taken during three-minute periods at ten- to fifteen-minute intervals and the average for the tested chicken was plotted on a graph (A in graph 1). From these data, a formula was calculated correlating the weight (W) of the chicken with its respiratory volume (Rv). It was found to be: $Rv = 0.78\ (\pm 0.12)\ W - 16\ (\pm 23)$ ml. per minute. This formula differs from Guyton's calculations [11] for small mammals: $Rv = 2.1\ W^{3/4}$ (curve B in graph 1).

For chickens, weighing less than 200 Gm., the two formulas give almost identical results but, above this weight, the chickens breathe comparatively more air than rabbits or guinea pigs of the same weight. This is probably due to a different method of breathing employed by birds; the air moves through their respiratory system in one direction [29] and does not reverse the direction of flow in the alveolar ducts from inspiration to expiration, as it does in mammals.

Determination of Newcastle Disease Virus in Feces.—Chickens were infected by intracrop instillation with 10^7 $e.l.d._{50}$ of the HP strain of NDV. Droppings were collected daily, weighed and mixed with 2.5 volumes of phosphate buffer (pH = 7.0), then homogenized in a Waring blendor, and centrifuged. The supernatant was treated with streptomycin (10 μg./1 ml.) and titrated for the presence of NDV in 10-day-old chicken embryos. After the death of the chicken, its lungs, brain, and intestinal contents were aseptically removed, weighed, separately homogenized, then treated as above, and the viral content determined.



Graph 1 — Correlation of respiratory volume of chicks with their weight.

Morbidity and Mortality of Chicks.—The infection of chickens was determined by clinical symptoms, respiratory and neurological. In most cases, the infection terminated in paralytic death. All birds which did not show any signs of the disease were bled on the ninth or tenth postexposure day, and the hemagglutination-inhibition (HI) titer of the serum determined by the *beta* procedure. On the fourteenth to sixteenth day, all surviving chickens were challenged intramuscularly with 10^3 to 10^6 e.l.$d._{50}$ of virulent NDV (HP) in order to detect those birds which, in spite of a negative HI titer, had become immune as a result of exposure. The infective dose for 50 per cent of chickens (c.i.$d._{50}$) was finally determined by the Reed and Muench method [22] and expressed in e.l.$d._{50}$. Similarly the 50 per cent lethal dose was calculated (e.l.$d._{50}$). In some experiments, upper or lower limits of infectivity were not reached. In such cases the results were expressed as more (+) than a certain value, or less (−) than a certain value.

RESULTS AND DISCUSSION

Intranasal Exposure.—The infective and lethal doses of NDV by various routes, as determined experimentally in chickens, are presented in table 3. The intranasal infective dose of both, the HP strain and the Haifa strain, was found to be about 5,000 e.l.$d._{50}$ for chickens of 30 to 70 days of age. For 7-day-old, parentally immune chicks this dose was apparently fivefold higher (strain HP, table 3, experiment 17). The comparatively large amount of virus required to infect a chicken by the intranasal route as compared to the intratracheal or aerosol-respiratory routes (see below) seems to be at first sight puzzling. It has been shown, however, by Bowers *et al.*[5] that when influenza virus or Serratia were intranasally instilled into mice, not more than 15 per cent of the inoculum could be later found in the lungs and, in many mice, no virus or bacteria could be demonstrated in the lungs. Similar results were obtained by Schechmeister [25] and Wells and Henle [32] when they infected mice intranasally with influenza virus. With viruses, however, one has to consider the possibility of disappearance of virus due to an eclipse phase after infection of the respiratory epithelium of the lungs.

Our own experiments performed on ether-anesthetized chicks with India ink showed that the instilled material mostly entered the esophagus and partly remained in the nasal passages and in the mouth cavity. Only in fully anesthetized chicks that exhibited no swallowing reflex was India ink found in the trachea, and then only a very small portion of it. The normal procedure of intranasal instillation of NDV vaccine is performed on waking birds; most of the instilled material is swallowed and only a part of it coats the nasal mucosa. No virus is expected to pass the laryngeal slit. It

TABLE 1—Determination of Concentration of Newcastle Disease Virus (NDV) in the Air During Aerosol Exposure of Chickens*

Exposure group	Total amount of NDV sprayed (e.l.$d._{50}$)	Volume liquid (VF) in impingers (ml.)	Sampling time (min.)	Total air passed (liters)	Relative humidity (%)	Recovery† of NDV in the air (%)	Results of titration‡			e.l.$d._{50}$ per ml.	e.l.$d._{50}$ per liter air (Cv)	Cv. E§
							Log dilution	Deaths of embryos	Log e.l.$d._{50}$			
							0.5	4/4				
		22.2	6	54.0	75	4.4	1.0	4/4	1.66	46	94	
							1.5	3/4				
B	6.1×10^5											95
							0.7	4/4				
		26.8	6	54.6	75	4.4	1.4	3/4	1.63	43	105	
							2.1	0/4				
							1.0	4/4				
C	5.0×10^6	24.7	5	46.0	70	6.2	1.7	3/4	2.63	427	1,140	1,250
							2.4	3/3				
							3.1	0/4				
							2.0	4/4				
		24.1	5	46.0	79	6.4	3.0	4/4	3.75	5,620	1.47×10^4	
							4.0	1/4				
							5.0	0/4				
D	6.0×10^7											15,000
							2.7	4/4				
		25.4	5	46.0	79	6.4	3.4	4/4	3.69	5,010	1.38×10^4	
							4.1	0/4				
							4.8	2/4				

* See experiment 10, table 3. † $\frac{\text{(Total amount of virus recovered from the air)} \times 100}{\text{Total amount of virus sprayed}}$ = percentage. ‡ Inoculum = 0.2 ml. § Concentration of NDV in titer of air (e.l.$d._{50}$) corrected for efficiency of sampling (E = 95 %).

may be assumed, therefore, that the dose required to infect chickens by the nasal route is high because the nasal mucous membrane is less susceptible to virus than is that of the lower respiratory tract or that the virus infects via the intestinal tract. It will be seen later that the second alternative is not acceptable as the amount of virus required to infect through the intestinal tract was found to be much higher than that which was sufficient to infect through the nose.

Aerosol Exposure.—By the aerosol route the chickens were infected when breathing 110 to 133 e.l.d.$_{50}$ (ages, 22 and 24 days). Results of a typical experiment in which respiratory-aerosol infection was measured are given in tables 1 and 2. The aerosol dose was thus found to resemble closely the intratracheal dose (see below) and to be 40 to 45 times lower than the intranasal dose. According to a previous study,[17] 100 e.l.d.$_{50}$ of Haifa virus vaccine were sufficient to immunize chicks by inhalation. Recalculation of quantitative data presented by Sinha, Hansor, and Brandly,[23] on aerosol exposure of chicks to strains of NDV of high or low pathogenicity but of similar pneumotropism, as well as the data of the same authors on results of aerosol exposure of chicks to the GB strain,[24] revealed them to be in close agreement with our findings.

Intratracheal Exposure.—The amount of virus required to infect 13- to 48-day-old chickens intratracheally ranged between 50 and 100 e.l.d.$_{50}$ This amount is comparable to that causing disease by inhalation. These results lend support to the hypothesis that the natural site of infection is the lower respiratory passages.

The results of a respiratory infection, aerosol or intratracheal, depend on the quantity of virus inhaled or instilled. When infection was initiated by limiting doses, its result was not always lethal. Some chickens developed respiratory symptoms, followed sometimes by paralysis, and finally recovered; in others, the infection could be detected only by a rise in the HI titer or by their resistance to intramuscular challenge with highly pathogenic NDV.

Intramuscular Exposure.—The infectious dose by the intramuscular route was found to be 70 to 90 times lower than that required by intranasal instillation (table 3, experiment 14, 14a). The incubation period of the disease was two to three days shorter after intramuscular inoculation than after intranasal instillation. These facts are important in considerations of desirability of either route of inoculation for efficient vaccination of flocks. Comparative studies on vaccination with live virus by intranasal instillation and intramuscular injection have been reported [3] and the conclusion was reached that the intranasal route was the more sensitive one. However, in our opinion such comparisons require two qualifications: (1) the quantity of virus introduced into the organism is important in determining the course of infection and the immune response—Hitchner [14] warns against a breakdown in immunity due to excessive exposure by the respiratory route with a resulting high mortality of chicks; and (2) data presented in this paper indicate a large dif-

TABLE 2—Determination of I.D.$_{50}$ of Newcastle Disease Virus (NDV) Strain HP by the Respiratory (Aerosol) Route in 24-Day-Old Chickens After a Twenty-Minute Exposure*

Exposure group	No. of chicks exposed	Ave. wt. (Gm.)	Ave. respiratory volume (ml./min.)	Exposed to e.l.d.$_{50}$ of NDV (Cv)	Total virus inhaled (e.l.d.$_{50}$)	Results of infection: No. of chicks: Sick and paralyzed	Died of NDV without symptoms	HI titer† of symptomless survivors	Susceptible to challenge‡	Ratio infected to exposed	e.i.d.$_{50}$§
A	6	110	70	10	13	—	—	All negative	6	0/6	
B	6	108	70	95	133	1	1	40	3	3/6	133
C	6	128	85	1,250	2,100	5	—	160	—	6/6	
D	6	115	74	15,000	21,000	2	3	80	—	6/6	
Control	....	125	80	—	—	....	....	All negative	6	.....	.....

* See also experiment 10, table 3. † Hemagglutination inhibition titer. ‡ 10^5 e.l.d.$_{50}$, intramuscularly, fourteen days after exposure. § Expressed in e.l.d.$_{50}$.

ference in infectivity of NDV (HP) when administered by different routes. Consequently, it appears desirable that the comparisons of various routes of vaccination with live virus be based on response to equal infectious doses. Up to now, what has been usually compared in published work, was the response to virus vaccines of equal titers. If, for instance, we had injected an equal amount of the HP strain (e.g., 4×10^4 e.l.d.$_{50}$) to 2 chicks, to 1 by the intramuscular and to the other by the intranasal routes, the first would have received about 1,000 infectious doses and the other only ten.

Comparison of susceptibility of parentally immune chickens to intramuscular injection of NDV revealed that 8-day-old chicks were much more refractory than 31-day-old chicks taken from the same flock (table 3, experiment 14 and 14a). The resistance of the younger group was 600 times higher than that of the older group, in confirmation of findings of Bornstein.[48] Similar experiments with parentally immune, 1-week-old chicks from another flock showed them to be less sensitive to NDV administered by the intranasal route (experiment 17). Comparison of the results of experiments 14 and 17 indicates a higher resistance of younger chicks to infection by both the intranasal (sixfold) and the intramuscular (eightfold) routes. It may be also seen that in spite of a similar age of chickens in experiments 14a and 17, those in 14a were much more resistant to intramuscular exposure. This was probably due to a different level of parental immunity present in chicks hatched from eggs obtained from different flocks.

Alimentary Exposure.— Alimentary infection could be initiated by doses of 20,000 e.l.d.$_{50}$ in chickens 34 days old. This result clearly places the alimentary route as the least sensitive one to the strain HP and probably also less important in the natural spread of the disease.

The use of drinking water as a vehicle for the vaccine virus has recently been advocated.[20] That such type of vaccination is very economical in saving of labor, needs no elaboration. The question remains, however, what should be the minimum concentration of virus vaccine in drinking water which would suffice to immunize chickens? Our findings indicate that this concentration should exceed 20,000 e.l.d.$_{50}$ in the volume of water which a chick is expected to drink in a few hours.

No quantitative studies have been reported on NDV excreted in feces. Van Waveren and Zuijdam,[29] working with various strains of Newcastle vaccine virus, demonstrated its presence in feces of vac-

TABLE 3—Comparison of Infectivity of Newcastle Disease Virus Administered to Chicks by Various Routes *

Experiment No.	Age of chicken (days)	c.i.d.$_{50}$/(e.l.d.$_{50}$) † by route				
		Intranasal	Intratracheal	Respiratory (aerosol)	Alimentary	Intramuscular
1	70	5,000	.....	.....	.....	.....
6	28	.....	.....	+50‡	.....	.....
8	22	.....	.....	110/(250)	+800	.....
9	13	.....	51/(+300)	.....	.....	.....
10	24	.....	.....	33/(435)	+3,000	.....
11	34	.....	.....	.....	20,000/(63,000)	.....
12	48	.....	100	.....	.....	.....
14	31	4,000/(4,500)	.....	.....	.....	45/(400)
14a	8	.....	.....	.....	.....	30,000
17	7	25,000	250	.....	.....	370
Ave. c.i.d.$_{50}$ (experiments 1 to 14)		4,500	75	121	20,000	45

* In all experiments, with exception of No. 1, HP strain was used. In experiment 1, Komarov's Haifa vaccine virus was employed. † The numerators express the infectious doses (50 %) for chickens in embryo lethal doses (e.l.d.$_{50}$). The denominators (in brackets) similarly express lethal doses for chickens. ‡ + = Titration end point not reached; more than the number stated.

cinated birds up to the nineteenth day after inoculation but quantitative data were not given. Our experiments showed that after alimentary exposure (intracrop instillation) the HP virus did not appear in feces for the first two days; it could be detected in the droppings from the third day until death of the chick (table 4). The concentration of NDV in feces reached its maximum on the sixth postinfection day (8×10^5 e.l.d.$_{50}$/1 Gm.). Examination of lungs, brain, and intestinal contents of the dead bird revealed the highest concentration of the virus (exceeding 6×10^5 e.l.d.$_{50}$/1 Gm.) in the lungs and fecal contents; the virus in the brain titered 4×10^4 e.l.d.$_{50}$/1 Gm.

TABLE 4—Elimination of Newcastle Disease Virus (NDV) in Feces of an Infected Chick*

Day after infection	Condition of chick	Wt. of collected feces (Gm.)	e.l.d.$_{50}$ per Gm. feces	Total NDV excreted (E.l.d.$_{50}$)
1	.....	22	—3†	.....
2	.....	15	—3	.....
3	.....	29	2,500	7.5×10^4
4	.....	25	25,000	6.2×10^5
5	Sick	10	71,000	7.0×10^5
6	Sick	13	800,000	1.0×10^7
7	Died	6	78,000	4.7×10^5

* Age of chick, 7 weeks. Infection with HP strain by intracrop instillation. † — = less than the number stated.

When a chicken was infected intramuscularly, the concentration of NDV in the feces reached its maximum earlier, *i.e.*, on the fifth day. In both cases, *i.e.*, after alimentary or intramuscular infection, at time of maximum excretion of virus, 25 mg. of feces would be sufficient to infect a chick via the alimentary tract. Feed or water contaminated with infective feces may thus become a potential source of infection.

Another source of NDV, which may contaminate feed or water, is the respiratory and nasal mucus of an infected bird.[29, 30] On one occasion, it was shown that a moribund chicken with individual water supply contaminated the water with NDV to the extent of 1,000 e.l.d.$_{50}$ per 1 ml.

Experimental evidence thus points to feces as a richer source of HP virus than mucous membranes of the upper respiratory passages; this point requires, however, more extensive studies. It is also known that NDV persists in feces for much longer periods of time than in water and has, therefore, better opportunities of reaching the air as dust after the drying of the feces than of staying alive in water.

The hypothesis that the alimentary route is of lesser importance in the natural spread of the disease appears to be strengthened by the findings of Walker and Powell,[30] who could not isolate NDV from feces of infected birds at any time during the disease, while the virus persisted in the upper respiratory tract for twenty-one days, thus favoring the aerogenic spread (droplet infection).

The quantitative results obtained in this study indicate that the natural portal of entry of the NDV is the respiratory system, particularly the lower respiratory passages (trachea, bronchi, and lungs); the smallest quantity of virus particles was required to infect chickens by this way. The relative sensitivity of chickens to infection by NDV (HP) by various routes follows the sequence: (1) intramuscular, (2) aerosol-respiratory, (3) intratracheal, (4) intranasal, (5) alimentary.

Summarizing, one may envisage the natural spread of ND as shown diagrammatically in figure 1. The main cycle of infection is: respiratory system to air to respiratory system; dried excreta and contaminated feathers contribute to air infection. The other cycle of infection may be: alimentary system to feces to feed and water to alimentary system. Not indicated in the diagram is the cycle: respiratory system to water and feed to alimentary tract; this although possible is not considered as very probable.

SUMMARY

The infectivity of a highly pathogenic strain of Newcastle disease virus (NDV) to chickens 3 to 6 weeks of age was quantitatively studied by the respiratory, alimentary, intranasal, and intramuscular routes.

The chickens were found to be most susceptible to infection by the intramuscular or respiratory (aerosol-breathing or intratracheal instillation) routes: approximately 100 e.l.d.$_{50}$ of the virus were required to initiate an infection.

The intranasal route required a forty-fold to fiftyfold greater amount of virus and 20,000 e.l.d.$_{50}$ were necessary to infect chickens by the alimentary route.

Parentally immune, 1-week-old chicks were found to be less susceptible to intramuscular injection or intranasal instilla-

tion of NDV, than the 3- to 6-week-old chicks.

Similar quantities of Haifa vaccine strain were required for aerosol and intranasal infection, respectively, as with the HP strain.

It is concluded that the main natural route of infection is the respiratory tract, the lungs being the most sensitive site.

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Infections with Mixed Etiology

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THE STATEMENT that tuberculosis is caused by the tubercle bacillus can be readily proved. We can see the organism in the affected tissue by special staining and can grow it in pure culture on artificial mediums. We can obtain it from all cases of tuberculosis. We can inject susceptible animals and produce the disease. From these experimentally diseased animals, we can again demonstrate the tubercle bacillus. These steps are the classical postulates of Koch, which he outlined as a set of rules to be followed in establishing the etiology of an infectious disease.

In some instances, we do not quite fulfill the requirements of Koch's postulates. For example, we are unable to cultivate the virus of hog cholera on artificial mediums.

We are sometimes inclined to accept an organism as the sole cause or primary cause of a disease on inadequate evidence. The ability to demonstrate an infectious agent in a particular disease by staining, culture, or serological means is indeed good evidence and strongly suggestive of the association. Swine erysipelas may be used as an example. In this condition, the organism *Erysipelothrix rhusiopathiae* can be demonstrated by staining techniques and culture from lesions with which it is unquestionably associated. However, all experimental attempts to regularly produce the field type of infection in swine have met with failure. In this connection, it must be recognized that the localized skin lesions, as experimentally produced with a culture in the manner first described by Dinter and Fortner (Wellman [1]), do not represent the variations of the naturally occurring disease of the field. With the evidence at hand, we can say that the organism is associated with swine erysipelas but we must admit we have failed in satisfying the most important of Koch's postulates, that is, producing the typical disease in the hog. In situations of this kind, it is obvious that we do not yet have adequate knowledge to give an explanation.

Kobe [2] reported a series of experiments in which he recognized the occasional occurrence of "inoculation erysipelas," but he showed that cultures isolated from these outbreaks were harmless to swine even in large amounts. He wrote about a virus which might cause a gastroenteritis and death from heart failure or very mild infection free of symptoms. This viral agent was believed by Kobe to prepare the way for infection with *Ery. rhusiopathiae*. He claimed to have readily transmitted swine erysipelas by using tissues of animals with natural infections which contained both the swine erysipelas organism and the hypothetical viral agent. While Kobe's claims have not been substantiated with respect to this relationship,[3,4] the European literature contains many references to the heart failure syndrome in pigs. The possibility remains that other factors play a part in swine erysipelas. A variety of laboratory animals can be artificially infected causing death from an acute disease thus fulfilling Koch's postulates so far as disease in these animals is concerned. Under these circumstances the organism undoubtedly is the sole cause.

In some instances, our imagination has been blunted and progress thwarted because of a general acceptance of a stated etiology of a given disease condition. The condition referred to as "colibacillosis" in calves will serve as an illustration. Most text books describe the condition and give the colon organism as the cause. Some text books [5] more cautiously indicate that *Escherichia coli* may be pathogenic at times or that certain strains may be pathogenic. And further, there is some question of *E. coli* being the primary etiological agent in white scours or calf scours. The organism has been repeatedly isolated from calves dead from this disease.

There have been some reports with evidence that serum from animals immunized

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with *E. coli* has a beneficial and protective effect. This has been construed to indicate support of the idea that *E. coli* is the cause of the condition. Since normal serum may have essentially as great an effect, the claim must be discounted. There has been considerable work done on strains of *E. coli* isolated from normal animals. Many commercial concerns have manufactured and sold products for this condition in calves, based on the assumption that the cause is *E. coli*. Much time and effort has been consumed in following these ruts with no stimulation of fresh thinking and progressive research on the disease. From the beginning of this theory, we have known that Koch's postulate for experimental production of the natural disease with the suspected etiological agent *(E. coli)* could not be fulfilled. Since *E. coli* can be found in the blood and tissues of animals with colibacillosis, we may suspect it of being responsible for some of the symptoms but we certainly lack proof of its being the primary cause. Some recent work [6] points to an understanding of this disease based on its experimental reproduction.

The comments on these two diseases are offered in criticism of our manner of thinking with respect to etiology of disease conditions. This is very important for our thoughts are a guide to our actions. If we erroneously accept a particular agent as the cause of a disease, our actions can be altogether out of line and we obtain no results from treatment or prevention. Perhaps more serious is the lack of a critical attitude in challenging the available information in these areas to see if it fits observations of the natural disease. If the information does not fit the naturally occurring situation, then we need constructive thinking so that progress can be made to obtain sound facts.

With our present knowledge, there are a number of disease conditions whose etiology is obscure. For at least some of them, we must consider the possibility of two or more etiological factors. Problems of this type are more difficult to approach. The remainder of this report will briefly review some examples of mixed etiology in order to demonstrate the variety of situations that do exist. These examples include a concerted action of a virus and bacteria, two different bacteria, a virus and a chemical compound, a virus and a parasite, bacteria and alteration of enzyme systems of the host, two different viral agents, and a virus plus a high environmental temperature. It is hoped that the discussion will stimulate thinking in this area.

The example selected of a virus and bacteria acting together is well known. Swine influenza was recognized as an entity in 1918 but its etiology remained obscure until 1931 when Lewis and Shope [7] and Shope [8] reported on the virus of swine influenza and a hemophilic bacillus found in animals with the disease. The virus alone would produce a very mild disease and the bacteria alone were almost nonpathogenic. However, together they would produce typical swine influenza. This disease is clearly the result of simultaneous action of two disease-producing agents. The transmission of the disease and its development, under natural conditions, remains to be fully explained. The hemophilic bacteria may be carried by many apparently normal pigs, and only the addition of the virus is needed to start the disease. Human influenza virus can enhance three bacterial infections. If guinea pigs are infected with human influenza virus, which alone is nonfatal, they could be killed with 1/250 the fatal dose of *Hemophilus influenza*, 1/100 the fatal dose of a hemolytic Streptococcus, or 1/10 the fatal dose of pneumococcus.[9] Perhaps influenza virus may enhance still other bacterial infections.

Fistulous withers of horses is an example of a disease caused by two kinds of bacteria. The cause of this condition was studied for many years and various opinions and ideas were advanced. Some believed that trauma was responsible; others believed that filarial parasites were responsible; and still others thought that Brucella organisms played a part in causing the condition. No one could experimentally produce the disease with any of the presumed causes. Rather recently *Actinomyces bovis* was isolated from an animal with this condition. Roderick, Kimball, McLeod, and Frank [10] made repeated inoculations of cultures of *A. bovis* into the supraspinous bursae of 12 horses, but no infection resulted. They also gave Brucella cultures in a similar way to 3 horses and these remained negative. A mixture of Brucella cultures and *A. bovis* cultures

was injected into the supraspinous bursae of 6 horses and 1 mule with the result that only 1 horse failed to develop infection while 1 animal developed a bursitis with recovery and 4 horses and the mule developed characteristic fistulous withers. From these experimentally produced infections, both organisms were isolated. Thus Koch's postulates were completely fulfilled and the etiology of the condition demonstrated.

Another example of different bacteria working together concerns mastitis. *Corynebacterium pyogenes* was commonly found in an acute mastitis of dry cows, but cultures of the organism were not highly pathogenic in experimental heifers. Reaction occurred in only four of 20 quarters exposed to *C. pyogenes*.[11] Stuart and coworkers [11] found *Streptococcus dysgalactiae*, an anaerobic coccus (probably *Micrococcus indolicus*), and a micro-aerophilic coccus mixed with *C. pyogenes* in secretions from cows with mastitis. By using a mixture of cultures of these four organisms in experimental heifers, they could produce the natural disease in 33 of 38 quarters thus exposed. Some attempt was made to study mixtures of only two kinds of bacteria but the number of trials was insufficient for distinct results. The effect of mixture of several cultures was clearly evident.

The cause of proliferative stomatitis and bovine hyperkeratosis was found to involve a viral agent and a chemical compound. Proliferative stomatitis was so commonly associated with bovine hyperkeratosis that it was regarded as part of the syndrome. Early work with bovine hyperkeratosis strongly emphasized the possibility of a viral etiology because of some of the features of proliferative stomatitis. Preliminary experiments in this direction were negative. After it was established that bovine hyperkeratosis was due to a type of poisoning (chlorinated naphthalene), there remained a need for satisfactory explanation of proliferative stomatitis. Test calves receiving the toxic substance which would cause bovine hyperkeratosis did not always develop proliferative stomatitis. It was found [12] that proliferative stomatitis was due to a virus-like agent which was without effect in older calves on normal feed, but would produce stomatitis in calves that had been receiving a hyperkeratosis-producing substance. Thus, it was necessary to bring two etiological agents or factors together before the complete disease condition as seen in the field could be produced. It was also found that very young calves were susceptible to action of proliferative stomatitis virus without the preliminary or associated action of the hyperkeratosis-producing substance.

The example of combined action of a virus and a parasite is drawn from experimental mice but is related to an infection of horses. The observations of lesions in the brains of horses due to larvae of nematodes diagnosed as virus encephalitis led to a study of larvae of *Toxocara canis* as a provoking factor in experimental Japanese B encephalitis of mice.[13] Experimental mice were given embryonated parasite eggs three or four days before they received a relatively light subcutaneous dose of the Japanese B encephalitis virus. The same amount of virus administered subcutaneously would not cause encephalitis or death. The mice receiving both embryonated eggs and virus died from acute encephalitis in a few days. In this instance, the migration of larvae in the brain of the mice activated an acute encephalitis from the later inoculation of virus. This may have been caused by the larvae destroying the blood-brain barrier or by creating a focus of lowered resistance in which the virus could gain entrance and grow. This example is somewhat different from the situation in which the lungworm of swine and earthworm may actually carry the virus of swine influenza.

A controlled infection in laboratory mice provides an example of alteration of metabolism influencing a bacterial infection. Interference with, or alteration of, the metabolism of the host at the time of infection provides an interesting area for study of the host-parasite relationship. Kreb's cycle of tricarboxylic acid metabolism is a well-recognized system of enzymic reactions. It can be inhibited by certain chemicals or some of the intermediary compounds can be introduced which will disturb the cycle. Berry and Mitchell [14] used mouse typhoid as their experimental infection with carefully measured doses of bacteria, and the chemicals were given in sublethal quantities. These workers found that the survival time of mice artificially infected with *Sal-*

monella typhimurium was reduced from an average of more than seventy-two hours to less than twenty-four hours. These workers also tested other pathogens of laboratory mice and obtained somewhat similar results with some variation depending upon the inhibitor or intermediary substance used.

An example of one virus preparing tissue for growth of a second virus has been known for many years. Belin [15] was able to cultivate foot-and-mouth disease virus in the skin of calves. Vaccinia virus and foot-and-mouth disease virus were inoculated simultaneously. The foot-and-mouth disease virus attached itself to the vaccinial virus lesions and, with successive animal passage, the foot-and-mouth disease virus became more and more attentuated. The virulence could not be restored. This finding has been the basis for development of one type of foot-and-mouth disease vaccine. This indicates that a normally resistant tissue could be made to support growth of a virus by infection with a second virus.

Perhaps a better example of concerted action of two viral agents exists in the case of louping ill of sheep. Evidence exists that infection of sheep with only louping ill virus would usually cause fever but no encephalitic symptoms. Symptoms of encephalitis regularly developed when the virus of tick-borne fever was also present with the virus of louping ill.[16, 17]

Griffin, Hanson, and Brandly [18] studied the effect of environmental temperature on the infection of mice with vesicular stomatitis virus. They found that the disease in mice which were acclimated to a low temperature had a longer incubation period and a lower mortality than in mice acclimated to a higher temperature. Factors such as this might be responsible for the absence of symptoms in certain types of mild infections.

If we will review our thinking on the etiology of disease in farm animals, we will eventually attain a more complete understanding. This will give us the knowledge to handle disease problems in a better manner. In our thinking and interpretation of the many results, ideas, and opinions concerning etiology, we can profitably depend upon the postulates of Koch laid down many years ago. The possibility of two or more factors being responsible for a disease was not specifically recognized by Koch; however, such a situation or the existence of enhancing agents for infection can readily be included in the process of experimental production of the disease condition. This requirement is perhaps the most important and significant. Some might say that experimental production of a given disease condition is difficult and perhaps impossible. We should be able to produce any disease which exists since its very existence is evidence of the fact that it can be done. Our inability to produce it when we wish simply means that we do not understand all of the factors involved.

Some idea of the sequence of events involved in a study of mixed etiology may be given by using listeriosis as an example. The natural infection in rodents can be readily produced and Koch's postulates fulfilled. The natural infection in cattle and sheep usually involves only the central nervous system and can not be regularly produced with the organism alone except by intracerebral inoculation. This has led to a search for other factors. By taking daily temperatures during an outbreak in sheep, a few sheep were found to have temperature elevations for two to three days with no other symptoms. The Listeria organism could not be isolated from such animals killed during the period of temperature elevation. However, in 1 sheep, the blood contained an agent which could be transferred to an embryonating chicken egg. This agent has been used in conjunction with Listeria in experimentally producing listeriosis in sheep.[19] While further work is necessary to clearly establish its relationship to the natural disease the lead is promising.

A variety of conditions have been described in this report to indicate the interplay of two factors in the causation of disease. There can be no doubt of the existence of other diseases with similar etiology. Many disease conditions occur in routine veterinary practice which have not been produced under experimentally controlled conditions. Veterinarians in general practice have a most unique opportunity to contribute ideas in this area. The factors responsible for disease conditions they encounter lie before them. Careful observation and thoughtful consideration of all the circumstances surrounding such a situ-

ation should lead to ideas on the cause. The ideas and suspected factors must then be challenged by experiments designed to test their validity. An inquiring mind with a critical attitude toward the etiology of many disease problems will form the foundation on which progress will be made.

It is hoped that this report will serve to stimulate discussion by veterinarians in practice and those in research. Each has much to contribute. Veterinarians in practice and farmers have problems for which our present knowledge is inadequate and research workers have areas in which they could suggest possible avenues for approach by field study. If the discussion will lead to an interchange of ideas, this report will have served its purpose.

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Distemper Complex in Wild Carnivores Simulating Rabies

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IN RECENT YEARS, rabies in feral carnivores has received considerable attention. Sylvatic rabies is considered to be a constant reservoir of the disease.[7, 21] Although aggressiveness and unusually bold behavior of naturally shy wild animals characterizes rabies,[20] such symptoms need not always indicate this disease. This communication reports outbreaks of canine distemper in gray foxes *(Urocyon cinereoargenteus)*, red foxes *(Vulpes fulva)*, raccoons *(Procyon lotor)*, and skunks *(Mephitis mephitis)*; the symptoms seen in these outbreaks were similar to those of rabies.[8, 9, 21]

REVIEW OF THE LITERATURE

Rabies has been a problem in wild animals in the past decade. The disease has been common among the feral members of the families Canidae, Mustelidae, and Procyonidae.[13, 15, 21] When animals of these families were reduced the incidence of rabies decreased.[2]

Goss,[8] however, pointed out that members of these families are susceptible to canine distemper, since all arose from the superfamily Arctoidea. Several outbreaks of canine distemper occurred among members of the families Canidae and Mustelidae confined at the New York Zoological Gardens.[8] Definite nervous derangement was observed in a red fox confined in another zoological garden. In this latter outbreak, intranuclear inclusion bodies were common in the epithelium of the urinary bladder; the intracytoplasmic type was uncommon.[1] However, intracytoplasmic inclusion bodies have been reported as common in the urinary tract.[22]

MATERIALS AND METHODS

All 35 specimens were feral carnivores taken within the Connecticut borders by conservation officers of the State Board of Fisheries and Game. Although the specimens represent a span of six years, the majority were taken during 1953. Species represented were the gray and red fox, the raccoon, and the skunk.

Living animals were chloroformed and blood was secured for hematological study. In all cases, cardiac blood and liver tissue were streaked on blood-agar plates; other grossly abnormal tissue was also subjected to bacteriological study. Portions of the brain and spleen were frozen at —20 C. for later inoculation into mice and ferrets.* A portion of the hippocampus was placed in a glycerin-saline solution (equal parts) and forwarded to a laboratory† where mice were inoculated intracerebrally and intraperitoneally and impression smears were made of the hippocampus.

For histological study, portions of the brain, semilunar ganglion, heart, lungs, kidney, urinary bladder, stomach, liver, duodenum, pancreas, jejunum, ileum, colon, adrenals, spleen, mesenteric lymph nodes, and tonsils were fixed in formol-saline. Duplicate portions of the hippocampus, semilunar ganglion, liver, kidney, and urinary bladder were placed in Zenker's fixative. Tissues were embedded in paraffin and stained by hematoxylin-triosin. Other stains such as Masson's trichrome, Levatidi's silver technique, periodic acid-Schiff, special Gram pyronin Y, and Williams' modification of van Gieson's stain were employed in selected cases. Fecal examinations were done by the flotation method. In a limited number of cases, detailed parasitological examinations were conducted.‡

Where fresh urine was obtainable, samples were examined by the dark field technique for Leptospira. Qualitative analyses were conducted for albumin, acetone, and sugar, and a sedimented portion was examined for cellular components.

RESULTS

Symptoms.—All the animals observed presented abnormal behavior (table 1). Eight were definitely aggressive; 1 raccoon attacked and bit a man, a second raccoon entered a barn and attacked a dog chained there, and a gray fox pursued and bit a horse. The remaining 5 aggressive animals attempted attack but unsuccessfully. All 35 animals were observed in populated areas in broad daylight and without fear of man.

A group of 7 animals had fallen into a deep sleep from which they could be aroused only with difficulty.

Convulsive seizures were a common manifestation; frothing at the mouth, snarling, and reckless snapping characterized these episodes.

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* Lederle Laboratories, Inc., Pearl River, N. Y., carried out the ferret inoculations.

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Raccoons were invariably affected by a purulent conjunctivitis, often suffering from complete closure of both eyes. Three members of this species manifested depraved appetite as 1 drank kerosene while another consumed paint; a third had a high chromium content (25 p.p.m.) in the feces.

The over-all clinical picture was typical of Steele's[20] comment on rabies: "The outstanding change in wild animals is their loss of fear of man and his environs."

Bacteriological Examinations.—Bacteriological examinations were generally without significant findings (table 1). *Pasteurella multocida* was isolated in a gray fox and in a raccoon, of which 1 case was considered to be canine distemper on histological grounds (table 1). In the other animal, histological evidence of canine dis-

TABLE 1—Incidence of Canine Distemper in Some Wild Carnivores

			Histological examinations				Other examinations							
								Hematology						
Species	Case No.	Neurological symptoms	Encephalitis	Hippocampus and semilunar ganglion	Cytoplasmic inclusion	Nuclear inclusion	Bacteriological findings	Erythrocytes (10^{-6}/cmm.)	Leukocytes (10^{-3}/cmm.)	Parasites	Ferret inoculations	Mice inoculations	Other observations	Diagnosis
Gray fox	1	+	+GC	—	—	—	—	6.5	10	C	.....	—	.....	Encephalitis
Gray fox	2	+	—	—	Bladder	Bladder	—	....	....	—	.....	—	.....	Distemper
Gray fox	3	+	—	—	Bladder	—	—	....	....	—	.....	—	.....	Distemper
Gray fox	4	+	+	—	—	—	—	....	....	—	.....	—	.....	Encephalitis
Gray fox	5	+	+	—	Bladder	Lymph node	—	....	....	—	Died	—	Interstitial pneumonia	Distemper
Gray fox	6	+	+	—	Bladder	Bladder	—	....	....	--	Died	—	Interstitial pneumonia	Distemper
Gray fox	7	+	+	—	—	—	—	....	....	A,C,T,	Died	—	.....	Distemper
Gray fox	8	+	+	—	—	—	—	6.5	7	A,G,T	.....	—	.....	Encephalitis
Gray fox	9	+	—	—	Bladder	Bladder	P	....	....	C,US	.....	—	.....	Distemper
Gray fox	10	+	+	—	Kidney	—	—	....	....	A,T	.....	—	.....	Distemper
Gray fox	11	+	+	—	Bladder	Bladder	—	....	....	A,C,US	.....	—	Toxoplasma in brain	Toxoplasmosis, distemper
Gray fox	12	+	—	—	—	—	—	....	....	A,T	.....	—	.....	No diagnosis
Gray fox	13	+	—	—	—	—	—	....	....	C	.....	—	.....	No diagnosis
Gray fox	14	+	+GC	—	—	—	—	....	....	C	.....	—	.....	Encephalitis
Red fox	15	+	—	—	Bladder	Bladder	—	....	....	—	.....	—	.....	Distemper
Red fox	16	+	+	—	Bladder	Bladder	—	....	....	....	.....	—	.....	Distemper
Red fox	17	+	+GC	—	Bladder	Bladder	—	....	....	....	.....	—	.....	Distemper
Raccoon	18	+	—	—	Bladder	Bladder	—	....	....	....	.....	—	.....	Distemper
Raccoon	19	+	+	—	—	Brain	—	7.8	28—	....	.....	—	.....	Distemper
Raccoon	20	+	+	—	—	Stomach	—	....	....	....	.....	—	25 p.p.m. Cr in feces	Distemper
Raccoon	21	+	—	—	Bladder	—	—	7.1	19—	....	.....	—	.....	Distemper
Raccoon	22	+	+	—	—	—	—	6.2	17	US	.....	—	.....	Encephalitis
Raccoon	23	+	—	—	Lung	—	—	4.7	17	....	.....	—	Interstitial pneumonia	Distemper
Raccoon	24	+	+	—	—	—	P	....	....	US	.....	—	Exudative pneumonia	Encephalitis
Raccoon	25	+	+GC	—	—	—	—	5.3	12	C,S,T,US	.....	—	Kerosene in stomach	Encephalitis
Raccoon	26	+	+	—	—	—	—	....	....	C,ET	.....	—	.....	Encephalitis
Raccoon	27	+	—	—	—	—	—	....	....	C,ET,US	.....	—	Interstitial pneumonia	Pneumonia
Raccoon	28	+	—	—	—	—	—	....	....	C,D	.....	—	25 p.p.m. Pb on liver	No diagnosis
Raccoon	29	+	+	—	—	—	—	....	....	C,D,T	Died	—	.....	Distemper
Raccoon	30	+	—	—	—	—	—	6.5	19	—	Died	—	Exudative pneumonia	Distemper
Raccoon	31	+	—	—	—	—	—	....	....	US	.....	—	Exudative pneumonia	Pneumonia
Raccoon	32	+	—	—	+	—	L	....	....	AC	.....	—	.....	Listeriosis, distemper
Raccoon	33	+	—	—	Bladder	Bladder	—	....	....	—	Alive	—	.....	Distemper
Skunk	34	+	+	—	—	—	—	6.3	10	—	Died	—	Interstitial pneumonia	Distemper
Skunk	35	+	+	—	—	—	—	9.3	15	A,C,G,T	.....	—	.....	Encephalitis

A = *Toxacara canis*; AC = *Ancylostoma caninum*; C = *Capillaria plica*; D = *Dracunculus insignis*; G = Gnathostoma spp.; GC = giant cells; L = Listeria; P = Pasteurella; p.p.m. = parts per million; T = *Taenia pisiformis*; US = *Uncinaria stenocephala*; ET = *Eurytrema procyonis*; Pb = lead; Cr = chromium.

temper was lacking and ferret inoculation was not done.

Listeria monocytogenes was isolated from a raccoon which was subsequently proved, by ferret inoculation, to have canine distemper. This organism had been previously isolated from a septicemic case in a wild raccoon [4] which was discovered dead.

It was believed in the present cases that canine distemper was the primary condition and that the discovered pathogens were secondary invaders.

Parasitic Examinations.—Parasites were present in a majority of the animals (table 1). Of external parasites, flea infestation *Ctenophalus canis* was noted in most foxes and raccoons. In addition, 1 gray fox had several ticks believed to be *Dermacentor variabilis.*

Internal parasites were present in a wide variety. The rarest of the lot was *Eurytrema procyonis* found in a recently captured raccoon.[14] The authors believed this worm to have been introduced into Connecticut by the importation of raccoons from southern states by hunting clubs. Literally thousands of the parasites were found in the lobular ducts of the pancreas. Their presence appeared to cause foldlike proliferation of the ductal epithelium (fig. 1). No histological alterations were observed in the pancreatic parenchyma although the urine revealed a moderate amount of sugar by Benedict's method. In addition, the Ross test for ketones was positive. The significance of the urine findings was veiled by the fact that 4 other raccoons also displayed sugar in the urine but never ketones.

Another unusual parasite of the raccoon for this area was *Dracunculus insignis.*[15] This was about 12 inches long and somewhat less than ⅛ of an inch in diameter. It was found in a tightly coiled position in the subcutis of the lateral aspect of the tarsus. Care had to be employed in the search for this parasite lest it be mistaken for a tendon. In some cases, a small weeping fistula over the site of the worm indicated its presence, but usually no external signs were present.

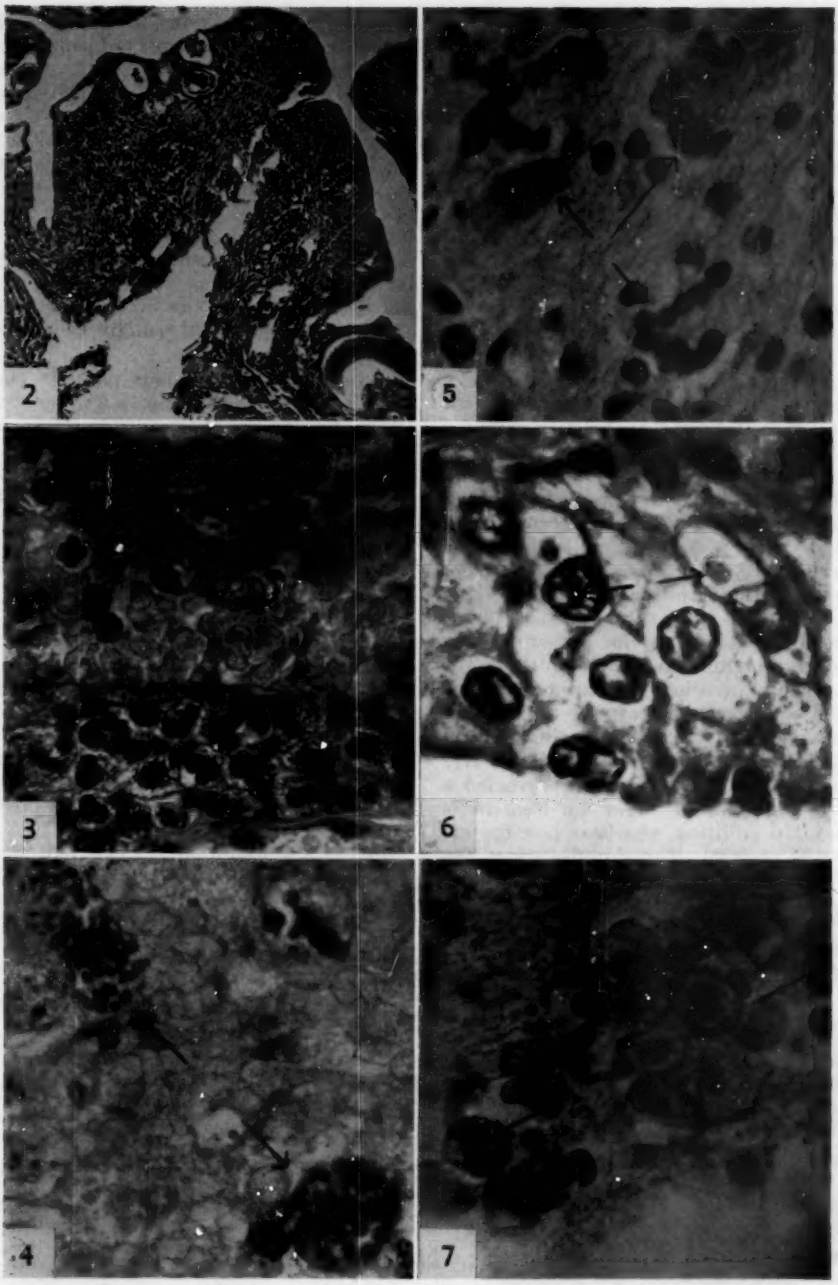
Capillaria was a common parasite (table 1). The species according to Penner [15] was either *Capillaria plica* or *Capillaria felis cati.* The characteristic double-operculated ova were usually found in the sediment of centrifuged urine. A urocystitis typified by roughened congested mucosa was the usual gross finding. Microscopic examination revealed hyperplastic renal epithelium containing larval forms of the parasite (fig. 2), while mature forms were seen on the surface of the mucosa. Aggregates of lymphocytes crowded the lamina propria.

A filarid considered to be *Gnathostoma sociale* was seen in the stomach of a skunk and a gray fox (table 1).

More common types of parasites such as *Ancylostoma caninum, Uncinaria stenocephala, Toxocara canis,* and *Taenia pisiformis* were frequently found (table 1).

Rabies Examinations.—Examinations for

Fig. 1—A pancreatic lobular duct from a raccoon contains an individual Eurytrema procyonis; the parasite is ingesting the epithelium of the mucosa. x 120.
Tissue embedded in paraffin and stained with hematoxylin and eosin.



All figures are photomicrographs of tissue embedded in paraffin and stained with hematoxylin-triosin.

rabies were negative in every case. None of the mice inoculated with suspensions of hippocampus died, and impression smears as well as paraffin-embedded sections of the hippocampus were negative for Negri bodies; no alterations were seen in paraffin-embedded sections of the semilunar ganglion. In the cases in which encephalitis existed it was not unusual to see alterations such as vascular cuffs and glial foci in the hippocampus. However, no corresponding changes were seen in the semilunar ganglion.

Pathological Examination.—A nonpurulent diffuse encephalitis of low intensity was a consistent finding (table 1). Of the 20 encephalitic cases seen, only 3 deviated from this pattern—2 raccoon brains exhibited perivascular cuffs with associated neutrophils (fig. 3), while the brain of a gray fox presented a large area of hemorrhage and cellular infiltration (fig. 4). This latter focus occupied the right corpus striatum and contained small cystlike bodies about 30 μ in diameter (fig. 5). The cysts consisted of smaller bodies about 4 μ in diameter which when stained by hematoxylin-triosin appeared to have a basophilic center surrounded by an acidophilic areola. These bodies failed to appear in sections stained by acid-fast, special Gram pyronin Y and periodic acid-Schiff stains, suggesting that they were not bacterial or fungal structures. On the basis of morphology,[10, 16, 17] these structures were considered to be Toxoplasma.

Of the remaining 17 encephalitic cases, 4 presented peculiar cellular aggregates which occurred in the cerebral white matter and, in 1 case, in the hippocampal gyri (fig. 5). They were glial in character but with concentrations of the nuclei to form ill-defined giant cells (fig. 5). No particular significance could be attached to these unusual formations.

The pathognomonic inclusion bodies of canine distemper[3, 6, 11] appeared to favor the epithelium of the urinary tract (fig. 6 and table 1). Some inclusion bodies were seen in such diverse tissues as lung, stomach, lymph node, and brain. Although the presence of intranuclear inclusion bodies in the lymph nodes suggested infectious canine hepatitis[19] the existence of intracytoplasmic inclusion bodies in the epithelium of the urinary bladder was considered to indicate canine distemper. In addition the pathognomonic intranuclear inclusion bodies of Rubarth did not appear in the hepatic cells.[19] The inclusion bodies seen in the brain (fig. 7) occurred in the nuclei of macroglial cells of the cerebral cortex and appeared to be the type described in so-called "hard pad" of dogs.[12]

A diagnosis of canine distemper was arrived at in 20 animals on the basis of (1) ferret inoculation with subsequent death or (2) the presence of pathognomonic inclusion bodies. The 15 remaining animals were classified as having encephalitis, pneumonia, or "no diagnosis" (table 1), since a positive identification of canine distemper was not possible on the basis of the above criteria. However, the authors were of the opinion that some of these 15 animals also had canine distemper. For example, ferrets died of canine distemper when inoculated with material from 4 animals neither presenting encephalitis nor inclusion bodies of distemper. This finding served to indicate that the absence of inclusion bodies or of encephalitis does not

Legends for Illustrations on Opposite Page

Fig. 2—A portion of the mucosa of the urinary bladder from a gray fox contains sagittal and cross sections of Capillaria. x 97.

Fig. 3—A cerebral arteriole from a raccoon is shown cut in the sagittal plane; the lower portion of the photograph is the vessel wall which is markedly infiltrated by granular leukocytes. x 877.

Fig. 4—A portion of the lentiform nucleus from a red fox contains in lower right and upper left cystlike structures, which in turn contain small two-toned bodies considered to be Toxoplasma (arrows). x 877.

Fig. 5—A section of the hippocampal gyrus from a raccoon showing glial nodules with nuclear concentration resembling giant cells (arrows). x 450.

Fig. 6—A portion of the renal epithelium of the urinary bladder from a red fox is shown; both intracytoplasmic and intranuclear inclusion bodies are present (arrows). x 877.

Fig. 7—A portion of the cerebral cortex, external granular layer, from a raccoon contains several intranuclear inclusion bodies (arrows). The pale various-sized bodies are serum casts. x 877.

preclude a diagnosis of canine distemper.

The diagnosis of canine distemper becomes more firmly established by the exclusion of rabies, the disease which was given prime consideration at the start of this series.

Other indications of disease were few. With the exception of a few cases of pneumonia some of which were distinctly interstitial in character (table 1), no alterations were noted. The excellent state of nutrition and the good condition of the coat suggested a short course of illness in these wild carnivores. This observation was in marked contrast to that of dogs in which canine distemper is often a chronic debilitating disease.[9]

SUMMARY

Thirty-five feral gray fox *(Urocyon cinereoargenteus)*, red fox *(Vulpes fulva)*, raccoon *(Procyon lotor)*, and skunk *(Mephitis mephitis)* specimens were presented with histories of aggressiveness, depraved appetite, or frequenting populated areas. All were captured in Connecticut. Examinations for rabies by means of hippocampal smears, paraffin-embedded sections of the hippocampus and the semilunar ganglion, and mouse inoculation of hippocampal material were noncontributory. Canine distemper was diagnosed in 20 animals based on either ferret inoculation or histological evidence of intracytoplasmic or intranuclear inclusion bodies which occurred chiefly in the urinary tract. In the remaining 15 animals characteristic inclusion bodies were lacking and ferret inoculations were not done; however, it was believed that some of these animals had canine distemper. This belief was based on (1) the similarity in the clinical history to that of proved cases, (2) the exclusion of rabies, and (3) the fact that distemper inclusion bodies are not always present in animals with feral distemper.

Twenty cases of encephalitis were found but not all were associated with distemper.

Bacteriological examination resulted in the recovery of *Pasteurella multocida* on two occasions and *Listeria monocytogenes* in one instance. These pathogens were considered secondary invaders since canine distemper appeared to be the primary illness in at least 2 of the animals.

Parasitological examination uncovered common invaders such as *Toxocara canis*, *Ancylostoma caninum*, *Uncinaria stenocephala* and *Taenia pisiformis*. *Capillaria plica* or *felis-cati* caused considerable urocystitis by its habit of burrowing into the bladder epithelium. *Dracunculus insignis* occurred in the legs of several raccoons. A rare find was the fluke *Eurytrema procyonis* in the pancreas of a raccoon. Toxoplasma spp. was identified in the brain of a gray fox on histological grounds. It was considered an opportunist, since canine distemper was definitely established in this case.

In feral carnivores rabid symptoms may apparently be caused by diseases of the distemper complex and are insufficient for diagnosis in a relatively rabies-free area. Complete histological examinations, in conjunction with mouse and ferret inoculations, are important diagnostic aids in the exclusion of feral rabies.

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Record Maintenance for a Long-Term Monkey Colony

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FARRIS [1] has pointed out that all the fine equipment on display in many laboratories will not aid in solving biological problems unless animals suitable for experimental purposes are available and the experimental animal's history and background are known. This fact has been given close attention in research programs conducted at the Radiobiological Laboratory of the University of Texas and the United States Air Force, where it is required that a *Macaca mulatta* monkey colony be maintained on a long-term basis. This is facilitated by a system of records presenting comprehensive information on both the colony and the individual animals. This paper describes the method which has evolved through trial and error to become a functional and economical system of recording information concerning the health of the animals in the monkey colony.

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MATERIALS AND METHOD

The lists and charts employed facilitate treatment of sick animals, assignment of animals to experiments, and compilation of research data. These lists and charts are concerned with specific information as described here.

Experimental Animal Assignment Chart.—This chart lists chronologically the experiments conducted and the animals involved in each. Cross-referenced with this chart is a numerical order list of animal numbers and their respective experiments. Minutes of interdepartmental conferences and memorandums concerning assignment serve as the source of this information.

This chart allows the veterinarian to know the experimental background of an animal prior to

INVENTORY & MORBIDITY CHART NOVEMBER 1954	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MONKEY															
COLONY STRENGTH	267	267	267	267	267	267	266	266	266	266	266	266	265	265	265
RECEIPTS															
SHIPMENTS															
DISEASE (TOTAL)	0	0	5	5	5	2	3	3	1	1	0	4	3	3	1
INTESTINAL			4	4	4	2	2	2	1	1		3	3	3	1
RESPIRATORY															
EXPERIMENTAL															
OTHER			1	1	1		1	1				1			
DEATH (TOTAL)							1						1		
DISEASE													1		
EXPERIMENTAL															
OTHER							1								
UNCOMMITTED	7	7	7	7	7	7	3	3	0	0	0	0	0	0	0

Chart 1—Example of inventory and morbidity chart used for record maintenance for monkey colony.

DATE	ANIMAL	PRESCRIPTION	SYMPTOMS	DIAGNOSIS	THERAPY
2 Feb'55	382	1 cc procaine penicillin daily for 3 days. 1 of 3 doses.	Laceration distal end of 3rd finger of right hand.	Skin and subcutaneous wound.	300,000 units procaine penicillin IM. Local infiltration with procaine. Sutured with 2-0 nylon. Stockinette bandage applied.
2 Feb'55	476	1 cc combiotic daily for 3 days. 1 of 3 doses.	Swelling and inflammation of gums.	Alveolar infection following extraction of rt.upper canine tooth.	Drainage established; 1 cc combiotic IM. (400,000 units procaine penicillin + 0.5 Gm. Streptomycin).

Chart 2—Example of medication record used for record maintenance for monkey colony.

clinical diagnosis and treatment, aids correlation of clinical symptoms and experimental procedures, and serves as a guide in further assignment of the animal.

The Weight Chart.—This record reveals the monthly weights of the individual animals. A mimeographed work sheet is used when monthly weights are taken, and these are then transcribed to the weight chart.

This chart aids in the evaluation of the colony's nutrition, in the detection of disease, and in the computation of therapeutic and anesthetic dosage.

The Inventory and Morbidity Chart.—This record (chart 1) gives the day-to-day census of the colony as to the number of animals on hand, the number clinically ill, and the type of illness (intestinal, respiratory, experimental, or other), the number of deaths and cause (intestinal, respiratory, experimental, etc.) as ascertained by necropsy, and the number of animals available for experimental use. The physical inventory count is taken every two months; in the interim, the addition or subtraction of receipts, deaths, and transfers maintains the inventory count. Medication records reveal the number of animals clinically ill and the clinical diagnosis; intra-office memorandums substantiate the number of deaths; the necropsy reports from the pathology group provide causative data. By means of this chart an observer has a relatively complete picture of the state of the colony for any day or month.

The Intestinal Disorder Prevalence Chart.—This chart lists those animals with diarrhea, showing occurrence by day of the month and the type of intestinal disorder (*i.e.*, hemorrhagic, mucoid, diarrheal, etc.). Animals with an intestinal disorder are determined from the daily clinical examination. This chart serves as a basic epidemiological tool in dealing with treatment of intestinal disorders on a colony-wide basis.

The Medication Record.—This record (chart 2) lists the symptoms, clinical diagnosis, and prescribed therapy for each animal requiring medication. This information is transcribed to the record of the individual animal.

The Daily Health Report.—This report, in the form of an interdepartmental bulletin, describes the general clinical appearance of each animal employed in an experiment. This information is obtained from the daily clinical examination and serves to apprise the interested departments of the state of health of their experimental animals.

The Individual Animal Record.—The information that appears on the charts, lists, and records is transcribed onto the record of the individual animal. Because of the variety of information and the irregularity with which it is received as to chronological order, it is necessary to prepare a rough draft before a permanent record is made. This is accomplished by the employment of a kardex file containing 5 by 8 inch cards, one for each animal, on which information is recorded as it is received. Insertions are placed in chronological order on these cards prior to transcription onto the permanent record.

The completed record in duplicate is permanently maintained in an identified flexible cardboard folder containing transfer data, experimental assignment, daily clinical examination results, symptoms, clinical diagnosis, therapy employed, results of fecal and blood examination, monthly weight, results of quarterly tuberculin test, x-ray negatives, pictures, circumstances of death, and necropsy report.

Supplemental information as to the procurement of the animal provides relatively complete knowledge of the animal's history from its capture in the jungle until its death.

DISCUSSION

The system described employs the reduction of data into accessible and easily read chart and list forms. There is duplication of information, but chart representation of often-needed specific information has been found to save much time and prevent confusion. The employment of a kardex file for rough draft entry and extraction of information on individual animals prior to final entry into the permanent record of the animal is of singular importance in the successful maintenance of complete individual monkey records.

The described charts serve as epidemiological source data in decisions involving mass-colony therapy. Information pertaining to individual animals during their maintenance at this laboratory, when coordinated with specific data involving experimental procedures, aids in the evaluation of experiments conducted with subjects from the monkey colony.

Differentiation Between Foot-and-Mouth Disease and Vesicular Stomatitis Viruses by Means of Mouse Inoculation

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Literature Cited

Cox and Olitsky[1] in 1933 demonstrated the susceptibility of adult Swiss mice, inoculated intracerebrally, to both "New Jersey" and "Indiana" types of vesicular stomatitis virus (VSV) of guinea pig origin.

In a subsequent publication, Olitsky, Cox, and Syverton[2] observed that if the intraperitoneal route was used only infant mice less than 14 days old could be successfully infected. Adult mice failed to be affected. Sabin, Olitsky, and Cox[3] studied in detail the susceptibility of young and adult mice to the virus of vesicular stomatitis (VS) injected by different routes. Nagel[4] showed the susceptibility of adult mice to a guinea pig-adapted strain of type "A" Vallée virus of foot-and-mouth disease inoculated intracerebrally. After 60 brain passages, the virus was found only in cerebral tissue.

With "O" and "C" types of virus, the results have not been satisfactory.[5] More recently, Skinner[6] demonstrated the susceptibility of suckling mice to the virus of foot-and-mouth disease.

For many years, the differential diagnosis of VS and foot-and-mouth disease was based on the inoculation of suspected material into different animal species, as horses, calves, pigs, and guinea pigs.[7, 8]

Brooksby[9, 10] showed that the complement-fixation reaction could be used to differentiate and type the viruses responsible for these two diseases. In certain instances, however, it has been found that field specimens, principally in the case of foot-and-mouth disease, may be negative for complement fixation but still show infectivity for the experimental animal. Virus may be present in quantities too small to be detected by complement fixation and still be infective. Since it is impractical and uneconomical to use cattle as a check on numerous negative complement-fixation reactions, particularly where diseases are enzootic, a simple, inexpensive test has been developed. The test was devised, primarily, to differentiate VS and foot-and-mouth disease viruses and is based on the susceptibility of adult and suckling mice to each of these viruses. In addition, the tissues from the inoculated mice may be used as antigens in the complement-fixation test to determine the type of the virus concerned.

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Materials and Methods

Foot-and-Mouth Disease Virus.—All strains were of Brazilian origin.

Type O.—Inoculums were prepared from two field strains, one from an outbreak in the State of São Paulo and the other from an outbreak in the State of Rio de Janeiro. The original strains were inoculated into susceptible calves to provide the necessary quantity of lingual epithelium (virus).

Type A.—Two strains were used. The first was a field strain from Rio Grande do Sul, passed once in a susceptible calf, and the other consisted of original material from an outbreak which occurred in Rio de Janeiro, Federal District. The material was sent to our laboratory in phosphate buffer glycerin without refrigeration.

Type C.—One of the two strains used was obtained from the Instituto Biológico of São Paulo where it is used as a seed virus in the preparation of vaccine. The other strain consisted of the original epithelial lesions from an outbreak in the State of Minas Gerais. These viruses arrived at our laboratory in phosphate buffer glycerin without refrigeration.

Vesicular Stomatitis Virus.—Since VS has not been identified to date in Brazil, all strains of virus used originated in other Latin American countries.

New Jersey Type.—One strain consisted of original epithelium from a natural outbreak in Panama. This strain had been preserved at —20 C. for several months before use. The second strain consisted of epithelium from the fifth bovine passage of the Huando strain from Peru. This had been stored at —20 C. for more than one year.

Indiana Type.—Two strains, designated "San Marcos Strain, XII passage" and "Carillo Puerto Vera Cruz Cosecha No. 995067 de 21/7/52" were used. These strains came from Peru and Mexico by courtesy of the Instituto Nacional Anti-Aftoso and Commissión Mexico-Americana de la Fiebre Aftoso, respectively.

Inoculums.—All inoculums were prepared by grinding 1 to 2 Gm. of lingual epithelium with sterile sand and resuspending the material in buffered saline as a 1:5 suspension. The suspension

was centrifuged at 2,500 r.p.m. for ten minutes in a horizontal centrifuge.

A portion of the supernatant fluid was mixed with an equal quantity of a penicillin and streptomycin solution so that the final virus suspension represented a 1:10 dilution of the original material. The concentration of antibiotics was calculated to provide 1,000 units of penicillin and 1 mg. of streptomycin per cubic centimeter of suspension. The suspension was allowed to stand in the refrigerator at 4 C. for a half hour after which it was checked for sterility by inoculating fluid thioglycolate medium.

Tenfold serial dilutions were prepared from each suspension beginning with a dilution of 1:10 of the original material. These dilutions were made with phosphate buffer solution.

Diluent.—A stock solution of phosphate buffer was prepared in accordance with the following formula:

$NaH_2PO_4 2H_2O$	2.6 Gm.
$Na_2HPO_4 12H_2O$	36.0 Gm.
Distilled water	1,092 ml.

This stock solution was diluted 1:20 with distilled water, as required, and sterilized in the autoclave. The pH range of the final diluent was 7.5 to 7.7.

Mice.—A Rockefeller Institute strain of Swiss mice was used throughout these experiments. The suckling mice were all 7 to 8 days of age and the adult mice were all 30 to 50 days of age, weighing 13 to 16 Gm. each. The weights of the adult mice were somewhat lower than normal due to the system of breeding wherein each mother suckles 8 infant mice instead of the usual 4 to 5. The normal weight at these ages would be 18 to 22 Gm.

Complement-Fixation Test.—The complement-fixation test used in these experiments was carried out according to techniques already described [11, 12] with the modifications indicated below. This technique uses 50 per cent end point readings utilizing a spectrophotometer.

Antigens.—Bovine tongue epithelium, muscle and brain tissues from the adult and suckling mice were the tissues used. A basic 1:5 suspension was made for titration and further passage and a portion reserved to prepare antigens for the complement-fixation test. Whenever possible two types of antigens were prepared from each suspension, one by extraction with chloroform (CLE antigen) and the other by high speed centrifugation (HSC antigen). Centrifugation was carried out in an international refrigerated centrifuge for thirty minutes at 16,000 r.p.m. with the aid of a multi-speed attachment.

Complement.—Four 50 per cent units of lyophilized complement prepared by The Texas Biological Laboratories were used in each test.

Antiserums.—Hyperimmune serums prepared in guinea pigs with standard types O, A, and C foot-and-mouth disease viruses and Indiana and New Jersey VSV were used. Some of the anti-foot-and-mouth disease serums were prepared in our laboratories, the remainder were obtained from The Virus Research Institute, Pirbright, England. The anti-vesicular stomatitis serums were obtained from the Animal Disease and Parasite Research Branch, Agriculture Research Service, U.S.D.A.

Procedure.—Each of the original inoculums was typed by the complement-fixation test and titrated in both suckling and adult mice. The tenfold serial dilutions of virus were inoculated intraperitoneally into groups of suckling mice and intracerebrally into adult mice, under light ether anesthesia. Each suckling mouse received 0.05 ml. of the suspension and each adult mouse 0.03 ml. The mice were examined twice daily for clinical signs of disease and for deaths.

Brain and muscle tissue were harvested from each group of mice which showed clinical signs of the disease and a separate 1:5 suspension was prepared from each tissue. This suspension was used for titration and to make further passages.

Serial passages of each strain were continued until clear-cut results were obtained in suckling and adult mice or by the complement-fixation test or by both methods.

TABLE 1—Experiments with Titration in Suckling Mice of Type O Vallée Foot-and-Mouth Disease Virus and Complement-Fixation Results

Strains and material used	Passage No.	Titer in suckling mice *	Complement-fixing reaction			
			Type of antigen	Serum O	Serum A	Serum C
Strain 1						
Bovine tongue		$10^{-5.6}$	HSC	85 **	0	5
Suckling mice						
Muscle	1	$10^{-4.9}$	CLE	100	0	0
			HSC	100	20	40
Brain	1	$<10^{-1}$	CLE	0	0	0
			HSC	0	0	0
Strain 2						
Bovine tongue		10^{-6}	HSC	100	20	48
Suckling mice						
Muscle	1	$10^{-4.2}$	HSC	100	0	4
Brain	1	$<10^{-1}$	HSC	0	0	0

* The same suspensions were titrated in adult mice and no virus was detected; HSC—high speed centrifugation; CLE—chloroform extracted; ** numbers represent the percentage of fixation. Negative results with both New Jersey and Indiana type serums not shown.

The 50 per cent end point was calculated by the Reed and Müench method based on death of inoculated mice.[12]

EXPERIMENTS AND RESULTS

TYPE O FOOT-AND-MOUTH DISEASE VIRUS

Strain 1.—Serial dilutions of virus infected bovine tongue epithelium ranging from 10^{-1} to 10^{-7} were inoculated into groups of suckling and adult mice. Eight mice of each age group were used for each dilution. A titer of $10^{-5.6}$ was obtained in the suckling mice but no deaths or signs of disease were observed in the adult mice. The bovine antigen gave positive fixation with anti-type O serum and a negative reaction with the other antiserums.

Antigens prepared from muscle tissue of the dead suckling mice showed strong fixation with anti-type O foot-and-mouth disease serum in the complement-fixation test while an antigen prepared from the brain tissue showed no fixation.

These antigens were titrated in suckling and adult mice. The muscle antigen showed a titer of $10^{-4.5}$ in suckling mice but the 10^{-1} dilution was not infective for adult mice. The brain antigen appeared to have no infectivity for either group of mice in a dilution of 10^{-1}.

Strain 2.—The same procedure was repeated with the second type O strain. The titer of the virus in suckling mice was 10^{-5} while adult mice showed no evidence of disease. The HSC bovine antigen gave strong fixation with anti-type O serum; however, some fixation with anti-types A and C serums was noted.

Antigens prepared from muscle tissue from the dead suckling mice gave clear fixation with anti-type O serum. Brain tissue antigens were negative (table 1).

TYPE A FOOT-AND-MOUTH DISEASE VIRUS

Strain 1.—Serial dilutions of virus-infected tongue epithelium gave titers of $10^{-2.5}$ in suckling mice and $10^{-1.5}$ in adult mice. The thioglycolate medium showed that the inoculum had not been sterile and these titers were probably inaccurate.

An antigen prepared from the original material gave clear complement fixation with anti-type A foot-and-mouth disease serum.

Muscle and brain tissue was harvested from both groups of mice for typing and titration. An HSC antigen from suckling mouse muscle gave 70 per cent fixation with anti-type A serum and slight fixation with anti-type O serum. The same tissue gave a titer of $10^{-3.5}$ in suckling mice. Brain tissue gave no fixation and a low titer of 10^{-1}.

A second passage was made in suckling mice, using a suspension of muscle tissue from the first passage. Brain and muscle tissues were again harvested. A CLE antigen prepared from the muscle tissue gave 37 per cent fixation with anti-type A serum. However, the HSC antigen showed strong fixation with anti-type A serum and slight fixation with anti-type O serum (36%) and anti-type C serum (15%). The muscle tissue gave a titer of $10^{-4.5}$. Three more passages were made in suckling mice in order to obtain a clearer result. Titration and complement-fixation test results are shown in table 2.

Strain 2.—The first passage of this strain showed a titer of $10^{-3.7}$ in suckling mice and was negative for adult mice. Strong fixation with anti-type A serum was obtained with an antigen prepared from the inoculum.

Muscle and brain tissue was harvested from the suckling mice for titration and complement fixation. Muscle tissue gave a titer of $10^{-5.3}$ and brain tissue a titer of $10^{-2.8}$. The CLE antigen from muscle tissue showed only slight fixation (20%) with anti-type A serum and no fixation with the other antiserums after the usual half-hour incubation. However, readings taken after fifteen minutes of incubation showed strong fixation with anti-type A serum while complete hemolysis was noted in the other serums and control tubes. The HSC antigen gave stronger fixation (81%) with anti-type A serum but some fixation was also observed with anti-types O (20%) and C (10%) serums. An HSC antigen from brain tissue gave no fixation.

Four more passages were made in suckling mice. Muscle and brain tissue was harvested from each passage for titration and typing. The CLE antigen from muscle from the second passage gave stronger fixation with anti-type A serum (50%) and no fixation with the other types. Similar results were obtained with material from the third and fifth passages. The HSC antigens gave stronger fixation with anti-type A serum but hemolysis was not complete with the other antiserums.

All the brain tissue antigens gave negative results. These results are summarized in table 2.

TYPE C FOOT-AND-MOUTH DISEASE VIRUS

Strain 1.—This strain produced a titer of $10^{-4.3}$ when inoculated into suckling mice but the adult mice showed no reaction. The inoculum gave strong fixation with anti-type C foot-and-mouth disease serum.

A CLE antigen prepared from harvested muscle tissue gave strong fixation with anti-type C serum (85%) and a very slight reaction (5%) with anti-type A serum. The HSC antigen prepared from this tissue gave almost the same results, there being strong fixation with anti-type C and very slight fixation with anti-types O and A serums. The brain tissue antigens gave negative results.

Strain 2.—The first passage was negative in adult mice and gave a titer of $10^{-3.3}$ in suckling mice. An HSC antigen prepared from the inoculum showed only slight fixation with anti-type C serum.

A suspension of muscle tissue from the first passage gave a titer of $10^{-4.3}$ and an HSC antigen prepared from it showed slight fixation (24%) with anti-type C serum.

The HSC muscle antigen from the second passage in suckling mice gave much stronger fixation (98%) with anti-type C serum and slight fixation with anti-type O and A serums (10% and 23%, respectively).

Two more passages were made in suckling mice. Antigens were not prepared with material from the third passage but muscle tissue was harvested from the fourth passage for titration and complement fixation. A titer of $10^{-5.3}$ was obtained and clear fixation was observed with anti-type C serum with both types of antigen (table 3).

TABLE 2—Experiments with Titration in Suckling Mice of Type A Vallée Foot-and-Mouth Disease Virus and Complement-Fixation Results

Strain and material used	Passage No.	Titer in suckling mice *	Complement-fixing reaction			
			Type of antigen	Serum O	Serum A	Serum C
Strain 1						
Bovine tongue		$10^{-2.5}$	CLE	0	100 **	0
			HSC	29	100	29
Suckling mice						
Muscle	1	$10^{-2.5}$	HSC	20	70	0
Brain	1	10^{-1}	HSC	0	0	0
Muscle	2	$10^{-4.5}$	CLE	0	37	0
			HSC	36	100	15
Muscle	3	$10^{-4.3}$	CLE	0	30	0
			HSC	6	70	5
Brain	3	$10^{-1.5}$	HSC	0	0	0
Muscle	4	10^{-5}	CLE	0	5	0
			HSC	27	100	36
Brain	4	10^{-3}	HSC	0	0	0
Muscle	5	$10^{-4.7}$	CLE	0	100	0
			HSC	10	100	20
Brain	5	10^{-3}	HSC	0	0	0
Strain 2						
Bovine tongue		$10^{-2.7}$	CLE	0	82	0
Suckling mice						
Muscle	1	$10^{-3.3}$	CLE	0	20	0
			HSC	20	81	10
Brain	1	$10^{-2.5}$	HSC	0	0	0
Muscle	2	$10^{-3.3}$	CLE	0	50	0
			HSC	10	65	0
Brain	2	$10^{-3.7}$	HSC	0	0	0
Muscle	3	10^{-5}	CLE	0	59	0
			HSC	40	100	17
Brain	3	$10^{-3.7}$	HSC	0	0	0
Muscle	4	10^{-4}	CLE	0	0	0
			HSC	11	41	17
Muscle	5	$10^{-4.7}$	CLE	0	48	0
			HSC	30	100	10
Brain	5	$10^{-3.7}$	HSC	0	0	0

* The same suspensions were titrated in adult mice and no virus was detected; HSC—high speed centrifugation; CLE—chloroform extracted; ** numbers represent the percentage of fixation. Negative results with both New Jersey and Indiana type serums not shown.

NEW JERSEY VESICULAR STOMATITIS VIRUS

Strain 1.—Serial dilutions of virus were inoculated into groups of 8 suckling and adult mice. Of the 8 suckling mice inoculated, only 2 died but in adult mice a titer of $10^{-4.5}$ was obtained.

Muscle and brain tissue was harvested from the adult mice. A suspension of brain tissue gave titers of $10^{-6.2}$ and $10^{-3.7}$ in adult and suckling mice, respectively, while a muscle tissue suspension gave titers of $10^{-4.7}$ in adult mice and $10^{-1.4}$ in suckling mice.

Both the CLE and HSC brain antigens gave 100 per cent fixation with New Jersey anti-vesicular stomatitis serum and no reaction with any of the other antiserums. Both antigens prepared from the muscle tissue of adult mice failed to produce fixation.

Strain 2. — A suspension of this strain did not appear to affect suckling mice but in adult mice 4 of the 8 inoculated with a 10^{-1} dilution of virus became infected and died. Brain and muscle tissue was harvested from the 4 dead mice.

Muscle tissue gave a titer of $10^{-3.7}$ in adult mice and $10^{-1.7}$ in suckling mice.

Brain tissue suspension gave a titer of 10^{-7} in adult mice and $10^{-4.6}$ in suckling mice.

Both CLE and HSC muscle antigens gave no reaction with any of the antiserums. Similar brain tissue antigens gave clear fixation with anti-New Jersey type serum (table 4).

INDIANA VESICULAR STOMATITIS VIRUS

Strain 1.—Serial dilutions of virus from 10^{-1} to 10^{-7} from bovine tongue epithelium were inoculated in suckling and adult mice. Titers of $10^{-1.7}$ and $10^{-2.4}$, respectively, were obtained.

Muscle and brain tissue were harvested from each group of mice and a 1:5 suspension prepared for titration and antigen. Each series of virus suspensions was titrated in suckling and adult mice.

Adult mice muscle gave titers of $10^{-5.5}$ and not less than $10^{-6.5}$ in suckling and adult mice, respectively. Adult mice brain showed titers of $10^{-6.4}$ and $10^{-6.7}$. Suckling mice muscle gave titers of $10^{-5.5}$ and $10^{-5.8}$ and suckling mice brain titers of $10^{-6.8}$ and not less than $10^{-8.5}$.

The HSC antigen prepared with brain from suckling mice, first passage, and the two CLE and HSC antigens prepared with brain from adult mice showed 100 per cent fixation with anti-Indiana vesicular stomatitis serum and negative results with the other antiserums.

The four antigens prepared with muscle from both groups of mice gave no fixation with all serums tested.

Strain 2. — Serial dilutions of bovine tongue epithelium, inoculated in groups of suckling and adult mice, gave the titers of $10^{-1.4}$ and $10^{-2.4}$, respectively.

Brains and muscles were harvested separately from dead mice of both groups and 1:5 suspensions prepared for antigens and titration.

TABLE 3—Experiments with Titration in Suckling Mice of Type C Waldmann Foot-and-Mouth Disease Virus and Complement-Fixation Results

Strains and material used	Passage No.	Titer in suckling mice *	Complement-fixing reaction			
			Type of antigen	Serum O	Serum A	Serum C Waldmann
Strain 1						
Bovine tongue		$10^{-4.5}$	HSC	0	0	100 **
Suckling mice			CLE	0	5	85
Muscle	1	$10^{-5.5}$	HSC	5	23	91
			CLE	0	0	0
Brain	1	$10^{-3.7}$	HSC	0	0	0
Strain 2						
Bovine tongue		$10^{-3.2}$	HSC	0	0	30
Suckling mice						
Muscle	1	$10^{-4.0}$	HSC	0	0	24
Brain	1	10^{-1}	HSC	0	0	0
Muscle	2	$10^{-5.2}$	HSC	10	23	98
Muscle	4	$10^{-5.2}$	CLE	0	0	97
			HSC	4	5	97

* The same suspensions were titrated in adult mice and no virus was detected; HSC—high speed centrifugation; CLE—chloroform extracted; ** numbers represent the percentage of fixation. Negative results with both New Jersey and Indiana types serums not shown.

Antigens from adult mice brain gave 100% fixation with anti-Indiana type serum. Muscle antigens were negative. The infective brain showed titers of $10^{-6.5}$ and $10^{-6.3}$ for suckling and adult mice, respectively. The muscle tissue gave $10^{-3.5}$ and $10^{-2.5}$ titers, respectively.

Brain and muscles from suckling mice, first passage, were harvested and 1:5 suspensions prepared for antigens and titration.

An HSC brain antigen and HSC as well as CLE muscle antigens showed 100 per cent fixation with anti-Indiana type serum and negative results with all other serums.

Brain tissue gave titers of 10^{-6} and 10^{-8} in suckling and adult mice, and muscle tissue showed a titer of not less than $10^{-6.5}$ in both age groups (table 5).

DISCUSSION

Due to difficulties of isolation, manipulation, and expense, the classical method of inoculating large animals for differentiation between foot-and-mouth disease and vesicular stomatitis is less and less favored. According to Traum,[14] it is sometimes difficult to obtain definitive results by this method, particularly when only one group of animals can be used. In addition, approximately seven days must elapse before a final result is obtained [15] and even then, in the case of foot-and-mouth disease, gives no indication of the type of virus which caused the outbreak.

On the other hand, the complement-fixation test can usually be relied upon to differentiate between vesicular stomatitis and foot-and-mouth disease viruses and between types and strains as well. However, a negative result does not always signify an absence of virus. For this reason the Mexico-United States Commission for the Eradication of Foot-and-Mouth Disease [15] adopted a diagnostic test which combined the complement-fixation test with large animal inoculations. The procedure consisted of a complement-fixation test on the original sample of lingual epithelium and the concurrent inoculation of at least 2 susceptible cattle with a portion of the same sample. If the first complement-fixation test gave negative results and the susceptible cattle developed vesicular lesions, it was possible to obtain fixation with epithelium from the inoculated animal. In this way, the Commission laboratory was able to increase the number of positive diagnoses of their results by 10 to 15 per cent. During a period of two years, the primary complement-fixation test confirmed the presence of foot-and-mouth disease virus in four samples and vesicular stomatitis virus in 308 samples of epithelium. Animal inoculations enabled them to isolate foot-and-mouth disease virus from a further 14 samples and VSV from 53 samples, thus increasing the total positive diagnoses from 312 to 379. It will be noted that the increase was particularly striking in the case of foot-and-mouth disease.

Our experience tends to confirm these observations which indicate that the complement-fixation test, alone, can not be relied upon entirely. However, in countries

TABLE 4—Experiments with Titrations in Mice of New Jersey Type Vesicular Stomatitis Virus and Complement-Fixation Reactions

Strains and material used	Passage No.	Infective titer in		Complement-fixing reaction		
		Suckling mice	Adult mice	Type of antigen	New Jersey serum	Indiana serum
Strain 1						
Bovine tongue		$<10^{-1}$	$10^{-4.5}$	CLE	86 *	0
Adult mouse						
Muscle	1	$10^{-1.4}$	$10^{-4.7}$	CLE	0	0
				HSC	0	0
Brain	1	$10^{-3.7}$	$10^{-6.3}$	CLE	97	0
				HSC	99	0
Strain 2						
Bovine tongue		$<10^{-1}$	10^{-2}	CLE	98	0
				HSC	96	0
Adult mouse						
Muscle	1	$10^{-1.7}$	$10^{-3.7}$	CLE	0	0
				HSC	0	0
Brain	1	$10^{-4.6}$	10^{-7}	HSC	96	0

CLE—chloroform extracted; HSC—high speed centrifugation; * numbers represent the percentage of fixation. All antigens gave negative results with foot-and-mouth disease types O, A, and C serums.

where one or both of these diseases is enzootic, the procurement of susceptible cattle becomes an almost insurmountable problem. The maintenance of special isolation facilities and the consequent high cost of animals render the inoculation of cattle impractical and uneconomic. The object of the present study has been to eliminate the need for large animals by substituting mice wherever feasible.

Henderson [16] has shown that mice, 3 to 4 weeks of age, are as susceptible as cattle to VSV when inoculated intracerebrally. Skinner [6] and Skinner, Henderson, and Brooksby [17] have also shown that suckling mice are almost as susceptible as cattle to the virus of foot-and-mouth disease. Seven of eight cattle strains of foot-and-mouth disease virus gave the same 50 per cent end point when titrated by intradermal inoculation of the tongue in cattle and simultaneously in suckling mice by intraperitoneal inoculation. Five of these eight strains were of recent field origin. Six titrations carried out in this laboratory in the same manner gave similar indications, the difference between the results in cattle and mice titrations being not more than log 0.5.

In this study, it was found that all ten strains of virus used were infectious for mice. None of the six strains of foot-and-mouth disease virus were infective for adult mice by intracerebral inoculation but all were infective for suckling mice by intraperitoneal inoculation. The low titers obtained with types A and C virus were probably due to lack of refrigeration during transportation.

On the other hand, VSV was pathogenic for adult mice by intracerebral inoculation but of low pathogenicity for suckling mice by the intraperitoneal route. This difference was most striking with the New Jersey type virus.

Investigations on the use of mouse tissue as an antigen for the complement-fixation test have shown that this material gives very satisfactory results. Muscle from infected suckling mice appears to contain a high concentration of foot-and-mouth disease virus while the brains of adult mice contain a high concentration of virus when infected by VS. In the case of foot-and-mouth disease virus, material from the first mouse passage gives definitive results with type O virus, a very high percentage of complement fixation being obtained. Types A and C do not give such a high percentage of fixation but further passages could be made until definitive results are obtained. Brain tissue antigen from suckling mice always gave negative results with anti-foot-and-mouth disease serum which agrees with the observations of Rohrer [15] who was unable to obtain fixation with infected mouse brains even after 1,300 passages.

TABLE 5—Experiments with Titration in Mice of Indiana Type Vesicular Stomatitis Virus and Complement-Fixation Results

Strains and material used	Passage No.	Infective titer in		Complement-fixing reaction		
		Suckling mice	Adult mice	Type of antigen	New Jersey serum	Indiana serum
Strain 1						
Bovine tongue		$10^{-1.7}$	$10^{-3.4}$	CLE	0	91 *
Adult mouse						
Muscle	1	$10^{-5.5}$	$10^{-6.5}$	CLE	0	0
				HSC	0	0
Brain	1	$10^{-6.4}$	$10^{-6.7}$	CLE	0	98
Suckling mouse				HSC	0	92
Muscle	1	$10^{-5.3}$	$10^{-5.8}$	CLE	0	0
Brain	1	$10^{-6.8}$	$10^{-8.5}$	HSC	0	100
Strain 2						
Bovine tongue		$10^{-1.4}$	$10^{-2.4}$	HSC	0	97
Adult mouse						
Muscle	1	$10^{-2.8}$	$10^{-2.8}$	CLE	0	0
				HSC	0	0
Brain	1	$10^{-6.5}$	$10^{-6.2}$	CLE	0	97
				HSC	0	97
Suckling mouse						
Muscle	1	$>10^{-6.5}$	$>10^{-6.5}$	CLE	0	98
				HSC	0	100
Brain	1	10^{-6}	10^{-5}	HSC	0	99

CLE—chloroform extracted; HSC—high speed centrifugation; * numbers represent the percentage of fixation. All antigens gave negative results with foot-and-mouth disease types O, A, and C serums.

Muscle tissue from adult mice infected with VSV failed to produce fixation although the tissue was known to contain an appreciable amount of virus. Brain tissue, on the other hand, yielded an antigen which consistently produced good fixation. This tissue showed a high concentration of virus. The Indiana strain of VSV which appeared to be more invasive than the New Jersey strain also gave fixation with an antigen prepared from suckling mouse muscle.

In general, a striking difference was noted between the results obtained with the two different types of antigen. While high speed centrifugation gave an antigen which showed strong fixation with homologous antiserums, a certain degree of nonspecificity was observed. In contrast, the chloroform-extracted antigen gave a clearer result but weaker fixation.

From these experiments, it would appear that mice may safely be used to replace cattle in the differential diagnosis of VS and foot-and-mouth disease. A routine diagnostic test has therefore been developed for suspect material and is conducted as follows:

A 1:5 suspension is prepared. A portion of this suspension is used in the complement-fixation test and the remainder treated with penicillin and streptomycin before intraperitoneal inoculation into suckling mice and intracerebrally into adult mice.

Mortality, in suckling mice only, indicates that the virus is that of foot-and-mouth disease whereas mortality in adult mice or both adult and suckling mice indicates VSV.

Antigens are prepared from brain and muscle tissue of all dead mice. Brain antigens are consistently negative by complement-fixation test with anti-foot-and-mouth disease serum but give a positive reaction with anti-vesicular stomatitis serum. Muscle antigen from suckling mice infected with foot-and-mouth disease will give positive fixation with the homologous antiserum.

Vesicular exanthema virus was not included in this study but to date mice do not appear to be susceptible to this virus.[18]

CONCLUSIONS

1) Foot-and-mouth disease virus is infective for suckling mice, 7 to 8 days old, inoculated intraperitoneally but shows no pathogenicity for adult mice 30 to 50 days old injected intracerebrally.

2) In suckling mice, dying after intraperitoneal inoculation of foot-and-mouth disease virus, muscle is much richer in virus than brain tissue. Antigens prepared from muscle give positive complement fixation with known serums. Brain antigens were in all instances negative.

3) The strains of vesicular stomatitis virus used appeared to be infective for mice of both ages. Adult mice inoculated intracerebrally seem to be more susceptible than suckling mice injected intraperitoneally. This difference is more marked with New Jersey strains. In one instance, virus could be isolated by intracerebral inoculation of adult mice while suckling mice remained normal.

4) In adult mice dying after intracerebral inoculation of vesicular stomatitis virus, brain contained more virus than muscle. In suckling mice, the difference of titers between brain and muscle is not so evident. Antigens prepared with brain either from adult mice or suckling mice gave positive complement fixation with known positive serum. Antigens prepared with muscle from adult mice were consistently negative while antigens prepared with muscle from suckling mice may or may not give positive fixation.

5) A test to isolate and differentiate vesicular stomatitis virus from foot-and-mouth disease virus by use of adult and suckling mice in combination with the complement-fixation test is practicable and economical.

SUMMARY

The previous work on susceptibility of mice to the viruses of vesicular stomatitis and foot-and-mouth disease is reviewed.

The authors describe experiments with two strains of each of the three classical types of foot-and-mouth disease virus and the two types of vesicular stomatitis virus. The pathogenicity of these strains for adult and suckling mice inoculated intracerebrally and intraperitoneally, respectively, was investigated as well as the use of infected mouse tissue as an antigen for the complement-fixation test.

It is suggested that suckling and adult

mice might replace large animals to a large extent in checking specimens which give negative results with the complement-fixation reaction.

A mouse test is outlined for the isolation and differentiation of vesicular stomatitis and foot-and-mouth disease viruses.

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Studies on *Pasteurella Multocida*. I. A Hemagglutination Test for the Identification of Serological Types

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IT HAS BEEN shown by means of a precipitin reaction that smooth or fluorescent strains of *Pasteurella multocida* possess a type-specific capsular antigen.[2] On the basis of serological differences in this substance, three types, A, B, and C were identified. An additional type, designated D, was demonstrated by precipitation and capsular swelling tests.[3]

It became evident after an extensive study of a large number of strains of *P. multocida* that, as routine typing techniques, the precipitation and capsular swelling tests had certain shortcomings.

In view of recent work on the adsorption of bacterial polysaccharides to erythrocytes and the subsequent agglutination of the treated red blood cells by specific antibody, it was thought that this procedure might lend itself to the identification of serological types of *P. multocida*. In this report a hemagglutination technique is described which has given results exceeding in specificity any of the previously employed typing methods. Some indication of the already widespread use of this technique can be gathered from Landy's[9] review of hemagglutination procedures.

The dissociation pattern of *P. multocida* has been referred to previously.[3,5,7] For practical purposes there are three principal colonial variants: (1) mucoid, (2) smooth or fluorescent, and (3) noncapsulated or rough. The principal characteristics of these three variants are summarized in table 1. Less frequent intermediate variants composed of sparsely capsulated organisms are seen often in avian cultures and laboratory strains that have been maintained for some time on artificial mediums.

Many cultures of *P. multocida* recovered from carrier animals and chronic processes in swine, poultry,[3,5] and rabbits are composed of predominantly mucoid variants. The capsules of the constituent organisms are large and are composed of hyaluronic acid.[3,4] These mucoid cultures are not typable by agglutination, precipitation, or capsular swelling techniques.[3-5]

TABLE 1—Some Characteristics of the Principal Variants of Pasteurella Multocida

Variants	Colonies on agar	Growth in broth	Mouse virulence
Mucoid	Large, flowing	Mucoid deposit	Moderate
Smooth (fluorescent)	Medium sized, discrete	Diffuse	High
Rough (blue)	Small, discrete	Autoagglutination	Low

MATERIALS AND METHODS

Preparation of Extracts of Pasteurella Multocida.—The 18- to 24-hour confluent growth from a blood-agar plate (7 to 8 per cent horse blood or a fresh beef infusion-agar plate) was removed with 4 ml. of normal saline solution. It was heated at 56 C. for thirty minutes to assist the removal of the capsular antigen. The organisms were then separated by centrifugation and the supernatant transferred to another tube.

Preparation of Human Type O Erythrocytes.—Sheep and chicken red blood cells were not satisfactory because they contain heterophil antigens as do many strains of *P. multocida*. Several samples of horse cells were also found to be unsatisfactory. The human O red cells were obtained through the courtesy of local hospitals. They were used with satisfactory results for periods up to four weeks after collection. The cells were stored in the original transfusion bottles and sufficient blood for three days was drawn off aseptically with a syringe. These samples were washed three times with 6 volumes of saline solution. The packed cells from the last washing were stored in the refrigerator and used as desired for a period of three days.

Sensitization of Erythrocytes with Extract.—To each bacterial extract consisting of approximately 3 ml., there was added 0.2 ml. of packed red cells. After thorough mixing, the extracts and cells were placed in the incubator at 37 C. for two hours. The red cells were then separated by centrifugation and washed once with 10 ml. of normal saline solution after which sufficient saline solution was added to give a 1 per cent suspension.

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The Diagnostic Laboratory of the Ontario Veterinary College and the Veterinary Laboratory of the Province of Alberta kindly submitted strains of *P. multocida*. The author thanks Mr. John Muysson for his technical assistance.

Preparation of Immune Serums.—The two serums, 1001 and 1305, were prepared in cattle and supplied through the courtesy of the Connaught Medical Research Laboratories. Six additional bovine serums were also available but were not used routinely. The other serums were prepared in rabbits. Satisfactory rabbit serums were usually obtained after approximately ten intravenous injections of suspensions killed with 0.25 per cent formalin. The organisms constituting the killed antigens were derived from young, blood-agar cultures. These organisms were washed in saline and then diluted in formalized saline until each milliliter contained approximately 10^9 cells. It was customary to give the first antigen injection subcutaneously. If sufficient titer was not obtained after the series of killed antigen injections, live cultures were administered subcutaneously. The dosage began at 0.25 ml. of a young broth culture and in some instances was increased gradually to 20.0 ml. All immune serums were absorbed by the addition of 0.2 ml. of packed, washed human cells to each 1.5 ml. of serum. After incubation for several hours, the cells were removed by centrifugation.

TABLE 2.—The Type Specificity of Strains of Pasteurella Multocida as Shown by the Hemagglutination Test

Serum	Culture extract	Reciprocals of serum dilutions					
		10	20	40	80	160	320
1001(A)*	1001(A)	+	+	+	+	+	±
1001(A)	1305(B)	—	—	—	—	—	—
1001(A)	361(C)	—	—	—	—	—	—
1305(B)	1001(A)	—	—	—	—	—	—
1305(B)	1305(B)	+	+	+	+	+	+
1305(B)	361(C)	—	—	—	—	—	—
361(C)	1001(A)	—	—	—	—	—	—
361(C)	1305(B)	—	—	—	—	—	—
361(C)	361(C)	+	+	±	—	—	—

* Serological type as determined by the precipitation test.

Performance of Hemagglutination Test.—Twofold serial dilutions of the immune type serums were made in saline in 12-mm. diameter tubes. The dilutions employed are listed in table 2. Routinely, 0.4 ml. of a 1 per cent suspension of treated red blood cells were added to the tubes containing 0.4 ml. of the serum dilutions. The two control tubes used were: (1) 0.4 ml. of 1 per cent suspension of treated cells and (2) 0.4 ml. of 1:5 dilution of the serum used plus 0.4 ml. of 1 per cent suspension of erythrocytes. After it was learned that a serum did not possess agglutinins for the human cells, the second control tube was omitted.

The racks of tubes were shaken vigorously then left at room temperature for approximately two hours, at which time a reading was taken. The tubes were then placed in the refrigerator and a second reading was made the following morning. Positive reactions were occasionally seen only at the second reading. This slower agglutination was attributed to a small amount of capsular antigen adsorbed to the red cells. To facilitate reading the test after two hours, the tubes were held over a well-lighted microscope mirror. A positive reaction consisted of marked agglutination, while a negative test showed no evidence of clumping. The following morning, the tests were read by shaking the tubes lightly to dislodge the cells. The cells were then allowed to settle and a reading was made over a mirror as described above.

Strains of Pasteurella Multocida Employed.—These are listed in table 3 according to their animal origin and other data. A number of strains could not be typed initially because of the absence of the capsular antigen. The type-specific capsular antigens of two of these strains, 1000(A) and 398(C), were restored by passage in mice. The noncapsulated variants are detectable on transparent agar by their failure to show fluorescence. They are referred to as intermediate or rough (*see* table 1). Freshly isolated strains are usually capsulated. Some avian strains, however, are either noncapsulated or are only sparsely capsulated on primary isolation. One or two mouse passages were not always successful in restoring the capsular antigen.

Absorption of Immune Serum with Bacteria.—To 1 ml. of serum to be adsorbed, there was added the confluent growth from one blood-agar plate. The organisms were removed from the plate with saline and collected by centrifugation. The mixture of serum and bacteria was incubated for several hours after which separation was accomplished by centrifugation.

RESULTS

In table 2 are presented the results obtained with three cultures, the serological identity of which had been established by other methods. Only cultures yielding a predominance of smooth, virulent variants were employed. The hemagglutination reactions confirmed in a highly specific manner the serological identity of the strains. A fourth serological type identified as D by precipitation and capsular swelling tests was also demonstrated by the hemagglutination test.

The greater number of strains of *P. multocida* isolated in the Ontario Veterinary College diagnostic laboratory have been of the mucoid variety. These cultures, on primary isolation, are composed for the most part of large slimy colonies, the organisms of which possess large capsules composed of hyaluronic acid. When these strains were examined by the hemagglutination test it was found that hemagglutination to a high titer occurred with all the bovine

type serums but not with any of the immune rabbit serums.

To demonstrate the serological distinction between the mucoid substance and the specific antigen, type serums 1000(A) and 1305(B) were absorbed with the markedly mucoid strain 323 of swine origin. The type-specific antibodies were not removed as both the absorbed serums agglutinated the red cells treated with the homologous, type-specific antigen. This experiment was repeated with the same results, employing for the absorptions two other mucoid strains.

In table 3, the strains of *P. multocida* examined in this study are listed according to their animal origin and serological type.

Types A and B were generally associated with acute pasteurellosis in cattle and buffalo. Type A strains predominate as the cause of fowl cholera. Only type C strains have been recovered from cats and dogs. Type D infections appear to be generally sporadic and have a wide host range. Although one culture from atrophic rhinitis was identified as type B previously,[8] all strains examined in this study from this disease have been either type D or mucoid. Noncapsulated cultures and mucoid strains, as previously observed, were generally associated with carrier states and chronic processes.

TABLE 3—Serological Type and Animal Origin of 122 Strains of Pasteurella Multocida as Determined by the Hemagglutination Test

	Types				Not typed		
	A	B	C	D	Mucoid	Non capsulated*	Total
Swine	0	0	0	7	31	0	38
Cattle	2	1	1	4	12	1	21
Poultry	17	0	0	2	1	16	36
Buffalo	1	2	0	0	0	2	5
Rabbit	0	0	0	0	3	0	3
Sheep	0	0	0	4	0	1	5
Goat	0	0	0	1	1	0	2
Man	0	0	1	3	0	3	7
Dog	0	0	1	0	0	1	2
Cat	0	0	2	0	0	0	2
Deer	0	0	1	0	0	0	1
Totals	20	3	6	21	48	24	122

* Composed of predominantly noncapsulated variants.

DISCUSSION

A further study of type cultures, kindly supplied by Roberts,[11] shows that his types I, II, III, and IV are identical, respectively, with types B, A, C, and D of this study. The original comparison of these types gave a somewhat different result.[3] Because of the considerably greater number of type D strains identified in this study as compared with previous studies,[3, 5] it is thought that some errors may have previously occurred in employing the capsular swelling and precipitation tests, with the consequence that some strains in reality type D were erroneously identified as type B. Errors, if such did take place, could be attributed to an insufficient appreciation of the dissociative complexity of *P. multocida*.

Of particular interest is the observation that red cells treated with the capsular hyaluronic acid of mucoid organisms were clumped by all the bovine type serums but by none of the rabbit serums. Even when almost totally mucoid strains were injected repeatedly into rabbits, no "antimucoid" antibody was demonstrable by the hemagglutination test. It may be that the doses of hyaluronic acid were sufficiently large to produce an "immunological paralysis." This phenomenon has been observed by a number of workers[10] with type-specific pneumococcus polysaccharides.

Many of the cultures examined appear to contain smooth variants as well as mucoid variants although the latter usually predominate. Because the mucoid substance is usually present in an amount larger than the specific antigen, the specific reaction is blocked. Some preliminary work has shown that this blocking can be prevented if the hyaluronic acid in the bacterial extract is depolymerized with hyaluronidase.

Now that considerable information is available on serological and immunological aspects of *P. multocida*, surely the value of the traditional bacterins and antiserums in the control of pasteurellosis should be reassessed. The recent work of Bain[1] has shown that conventional bacterins are without value in the prevention of acute systemic pasteurellosis in cattle. Dougherty[6] has demonstrated the superiority of the chicken embryo vaccine to broth bacterins in the prevention of fowl cholera.

SUMMARY

A hemagglutination test is described for the identification of serological types of *Pasteurella multocida*. This technique

yields highly specific reactions and is considered to have advantages over other typing methods. The specific capsular antigens and the hyaluronic acid capsular material were extracted with saline and adsorbed to human type O erythrocytes. To these treated cells were added appropriate dilutions of the specific typing serums. Positive reactions consisted of agglutination of red cells. One hundred and twenty-two strains of *P. multocida* from various geographic sources and animal species were typed by the hemagglutination test. Four different serological types, A, B, C, and D, were recognized. A large number of mucoid strains and noncapsulated cultures which were not typable were also found. The serological results are discussed briefly in relation to the nature and occurrence of pasteurellosis in the various species.

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Helminth Parasites and Neoplasia

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DURING the last fifty years, papers have appeared from time to time which have attempted to demonstrate associations between helminth parasites and cancer. The most recent of these was a report in this journal [1] of 10 cases of malignant esophageal tumor in the dog and their possible relationship to *Spirocerca lupi* infection. As this subject was reviewed in 1933 [2] and again in 1937,[3] the present discussion will confine itself for the most part to the literature of the last twenty years.

HISTORICAL BACKGROUND

Although some of the larger helminths were known to the earliest civilizations, it was not until the fourteenth century that they first were associated with disease. In 1379 de Brie, a shepherd-veterinarian, discovered that a liver fluke *(Fasciola hepatica)* was the cause of necrotic liver disease (black disease) in sheep.[4] This epochal event marked the birth of the science of parasitology and, of our knowledge, of the causality of infectious diseases. During the seventeenth and eighteenth centuries many other pathological conditions were ascribed to helminths, some of which are now known to be caused by microorganisms. Following this initial flourish, scientific interest in helminthology was virtually eclipsed during the nineteenth century by the spectacular advances made in microbiology. In human medicine, helminthiases came to be considered diseases peculiar to the tropics. The discovery of arthropod transmission of filariasis [5] helped to revive interest in the helminthic diseases, but perhaps the most significant contribution to the rebirth of helminthology was the discovery by the Italian veterinary pathologist Perroncito [6] that hookworms were the cause of the epidemic anemia of European miners. This recognition that helminths were capable of producing severe disease in man, as well as in his domestic animals, under temperate conditions created new interest in helminth pathogenesis.

With other advances in the histopathology of the helminthiases came the discovery by Curtice [7] that intestinal nodular disease of sheep was caused by a nematode, *Oesophagostomum columbianum*, and was not a form of tuberculosis as had previously been supposed. This and other observations that helminths in tissues were responsible for reactive cellular proliferations of various types prompted speculation upon the oncogenic propensities of the metazoan parasites. The reactive granulomata characteristic of a variety of helminthic diseases, and such microbial diseases as tuberculosis, glanders, and actinomycosis, were studied in an attempt to relate them to neoplastic processes.

Borrel in 1901 [8] discussed a parasitic theory of cancer, and in 1906 [9] he reported on an observed coincidence of hepatic sarcoma in the rat and the presence of the larvae of the tapeworm *Taenia taeniaeformis*. Previously a filtrable agent had been shown to be the cause of contagious myxomatosis of rabbits.[10] These discoveries, soon followed by reports on the transmission of fowl leukosis [11] and spindle-cell sarcoma [12] by cell-free filtrates, provided the initial impetus to the development of a field of experimental oncology.

Since then more than 40 helminths [3] have been incriminated as oncogenic agents. Most reports may be dismissed for the following reasons (*see* Bogliolo [3] for this literature): (1) no histopathological evidence, (2) reactive granulomata with no evidence of neoplasia, (3) purely coincidental occurrence (for example, trichinae in rhabdomyomas). The several relationships discussed here are those of actual or potential importance or are otherwise of current interest.

GONGYLONEMA NEOPLASTICUM

This nematode (spirurid) parasite, as an adult, inhabits the gastric mucosa of the rat. Known intermediate hosts include various coprophagous and meal beetles and cockroaches.

It was described originally by Fibiger [13] as being associated with what he considered to be cancerous growths in the gastric mucosa of the rat. The studies [14] by Fibiger and his associates with this parasite were among the first experimental approaches to the cancer problem. Their importance, widely recognized at the time, led eventually to a Nobel Prize. The criteria for malignancy observed by these workers, however, were early questioned publicly.[15]

Brumpt [16] examined 750 wild rats, 300 of which he found to be naturally infected with *G. neoplasticum*. Eighteen of these had hyperplastic growths in the gastric mucosa, and only 1 had an associated gastric carcinoma.

Attempts to repeat Fibiger's work [16–19] have been unsuccessful. Passey *et al.*[17] were able to induce gastric hyperplasias only in rats fed on a white

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bread diet (this diet was used by Fibiger to maintain his rats). These reactions were enhanced by infection with *G. neoplasticum*, but none of the growths were carcinomatous. Klein and Palmer,[19] in a review on experimental gastric carcinoma, expressed the belief that a vitamin A deficiency very likely resulted from the bread diet under the conditions of Fibiger's work; therefore, they discounted somewhat his findings. (*See* Tannenbaum and Silverstone[20] for a discussion of nutrition and cancer.) Recently, Hitchcock and Bell[18] attempted to repeat Fibiger's results. They were able to demonstrate in carefully planned experiments that lesions, similar to those Fibiger produced, occurred in their experiments only in rats on a vitamin A-deficient diet. They also showed that while *G. neoplasticum* affects the gastric mucosa of the rat as a chronic biological irritant, examination of Fibiger's original slides, as well as their own material, failed to reveal growths considered by them to be carcinomatous.

It must be concluded, therefore, that, while *G. neoplasticum* acts as a biological irritant, it has not been demonstrated capable of inciting malignant growths, when the growths are judged by present day criteria for malignancy.

TAENIA TAENIAEFORMIS

This cestode parasite, as an adult, inhabits the intestinal lumen of the cat and dog. The rat serves as an intermediate host; larval parasites (cysticerci) develop within cysts in the liver.

Borrel[9] first observed a coincidental occurrence of hepatic sarcoma in the rat with the presence of larval *Taenia taeniaeformis (Cysticercus fasciolaris)*. Bullock and Curtis[20a] subsequently induced these neoplasms experimentally by infecting rats with eggs of this parasite. To date more than 7,500 Cysticercus tumors have been experimentally produced.[21] Most of these have been sarcomas, forty-nine being bone-forming tumors, while a few have been of other histological types.[21, 22] The tumors appear to arise from the fibrous capsule formed by the host about the larvae and not from the liver tissue itself.[20a] Both cysts and tumors have been successfully propagated in tissue culture.[23, 24] Generally, malignant tumors arise only following an infection of from 250 to 675 days' duration. Dead or dying parasites have not been responsible for the malignant irritation; healthy, living larvae have been found within the sarcomatous cysts. Increased susceptibility to the Cysticercus-induced tumors has been demonstrated in certain strains of rats,[25] as has also age susceptibility, most of the sarcomatous rats being between 13 and 19 months of age. Peritoneal metastasis of these tumors has been a common feature, and metastases to other organs have also occurred.[26]

Dunning and Curtis[27] have recently shown that the intraperitoneal injection of suspensions of ground, washed Taenia larvae into uninfected rats produces multiple peritoneal sarcomas and mesotheliomas in 23 to 787 days following injection. The active substance appeared to these authors to be associated with the calcium carbonate corpuscles normally present in the body fluid of cestode larvae.[27, 28] Attempts to isolate a specific active agent by centrifugation and filtration of larval suspensions have not as yet been successful.[28, 29]

SCHISTOSOMA HAEMATOBIUM

This trematode parasite, as an adult, inhabits the venules of the bladder, mesenteries, and pubic region of man and monkeys. Ova usually migrate through the bladder wall and pass out with the urine. Various gastropod mollusks serve as intermediate hosts.

Ferguson[30] had early associated cancer of the urinary bladder in man with the presence of schistosome eggs *(S. haematobium)* on the basis of pathology of 40 cases. Other observers had previously suggested such a possibility.

Many subsequent reports have claimed or assumed a causal relationship between *S. haematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum* and human cancer; and *Schistosoma bovis* and bovine cancer.[3] Brumpt[31] concluded on the basis of statistical evidence that there is a very close association between *S. haematobium* infection and the urinary bladder neoplasms in man. Afifi,[32] on the other hand, felt that although the assumption of such an association would be difficult to avoid (particularly in countries like Egypt where the schistosomiasis attack rate is from 30 to 90 % of the population) no one has yet demonstrated that the incidence of cancer is greater in a bilharzial population than in a like, but uninfected, population. He concluded that, until this is shown to be the case or a definite carcinogenic agent or predisposing factor found, the use of the term "bilharzial or schistosome cancer" is unjustified.

SPIROCERCA LUPI

This nematode parasite, as an adult, inhabits the esophageal and aortic walls of the canine species. Coprophagous beetles serve as intermediate hosts; various frogs, snakes, lizzards, birds, and mammals may function as larval transport hosts.

No reference can be found prior to that of Seibold *et al.*[1] which associates the canine spirurid, *Spirocerca lupi*, with neoplasia. Pronounced reactive proliferations in the aortic and esophageal walls, as a result of infection with this parasite, have been noted by most observers, but no evidence of neoplasia or metastasis has been recorded.

In histopathological studies of canine spirocercosis Hiyeda and Faust,[33] Hu and Hoeppli,[34] and Barron[35] found that some ossification occurred in aortic lesions with no evidence of such changes in the esophagus. Since Hoeppli[2] was interested at that time in the association between helminths and neoplasia, it seems unlikely that the condition

reported by Seibold *et al.*,[1] if present, would have escaped his notice.

Jackson,[26] in his monograph on neoplasia in domestic animals, mentions but does not describe one canine esophageal fibrosarcoma among 72 canine neoplasms in the Onderstepoort (South Africa) collection. The rarity of primary esophageal tumors in the dog (Mulligan[27] records one carcinoma and one leiomyoma), and the presence of canine spirocercosis in South Africa[28] suggest that Jackson's case might possibly have been a Spirocerca sarcoma.

Yoshida,[29] in describing esophageal gnathostomiasis (*Gnathostoma spinigerum* infection) in Japanese mink, a condition somewhat similar to canine spirocercosis, remarks on the almost constant occurrence of calcareous areas in the reactive esophageal granulomas produced. However, as far as could be determined by the reviewer, no evidence for neoplastic association has been reported for *G. spinigerum* in any host.

The series of esophageal tumors reported by Seibold *et al.* have all been fibrosarcomas and osteosarcomas. Their malignant nature has been firmly established, and several instances of metastasis have been recorded. Although these tumors have not as yet been induced experimentally, their association with the helminth is altogether different than that, for example, which exists between carcinoma of the bladder in man (a relatively common neoplasm) and schistosomiasis, in many areas a common infection. By contrast, while canine spirocercosis is not uncommon, canine esophageal tumors of any type are rare, and primary bone-forming tumors of the esophagus are practically unknown in any species. The series of reported tumors, however, all apparently occurred in association with this parasite. It is somewhat difficult to explain why this neoplasm has not been previously observed unless important factors other than (or in addition to) the parasites are involved in Seibold's series of Spirocerca sarcomas.

DISCUSSION

Helminths in host tissues present a complex host-parasite relationship. Many are not dependent upon blood, lymph, or phagocyte transport, or on colonial growth for movement within the host but, instead, migrate through intact tissues and organs where extensive damage may be produced. Because of their large size and anatomical complexity, helminths escape engulfment by even the largest of the host's giant cells. Somatic tissues of the parasite act as foreign proteins and, as a consequence, eosinophilia, dermatitis, edema, and other allergic manifestations are common to infections with many tissue-invading helminths. Protective antibodies are elicited from the host both in response to somatic antigens and antigens in the secretions and excretions of the parasite. The extent to which these reactions occur usually depends upon the degree of tissue intimacy of the parasite.

As a helminth becomes less active in its host's tissues, it renders itself more susceptible to encystment by the defensive cells of the latter. Such is the case when certain adult helminths, such as *Spirocerca lupi*, oviposit in specific tissues or organs, and when *Trichinella spiralis* and various tapeworms spend a portion of their larval life encysted in the tissues of their host. In some instances the host's cellular response is mild, as in the case of porcine trichinosis, while in other instances it may be extensive, as in reported instances of parasitic sarcoidosis of man.[40] In addition, the reactions in immune or abnormal hosts may differ considerably from those encountered in the usual host. In murine cysticercosis *(Taenia taeniaeformis)*, canine spirocercosis, and possibly other helminthiases, this cellular reaction of the host may assume malignant characteristics.

On the basis of the available literature regarding neoplasms ostensibly associated with helminthiases this reviewer concludes that only one causal relationship, that between larval *T. taeniaeformis* and sarcoma in the rat, has thus far been satisfactorily established. A similar relationship is strongly suggested between *S. lupi* and sarcoma in the dog although experimental proof is lacking in this instance. The association between neoplasia and schistosomiasis seems to have some statistical basis, but the biological relationship of cause and effect remains to be established.

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